İSTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INVESTIGATION OF CYP17A1 AND CYP19A1 GENE EXPRESSION LEVELS AND AROMATASE ACTIVITY IN INVASIVE DUCTAL BREAST CANCER TISSUES

Ph.D. THESIS

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Department of Molecular Biology – Genetics and Biotechnology

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

DUKTAL MEME KANSERİ OLGULARINDA CYP17A1 VE CYP19A1 GEN BÖLGELERİNİN EKSPRESYONLARININ VE AROMATAZ AKTİVİTELERİNİN İNCELENMESİ

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OCAK 2017

Mete Bora Tüzüner, a Ph.D. student of ITU Graduate School of Science Engineering and Technology student ID 521082062, successfully defended the thesis entitled "Investigation of CYP17A1 and CYP19A1 Gene Expression Levels and Aromatase Activity in Invasive Ductal Breast Cancer Tissues", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To the Ph.D. warriors who never give up the fight even they fall,

FOREWORD

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TABLE OF CONTENTS

Page

ABBREVIATIONS

A : Androstenedione
ACN : Acetonitrile
AR : Androgen Receptor
BCA : Bicinchoninic Acid
BMI : Body Mass Index
BRCA1 : Breast cancer 1
BRCA2 : Breast cancer 2
BSA : Bovine Serum Albumin
C/EBPα : CCAAT/enhancer-binding protein alpha
cDNA : Complementary DNA
CYP17A1 : Cytochrome P450 17A1
CYP19A1 : Cytochrome P450 19A1
DCIS : Ductal Carcinoma Insitu
dH_2O : Distilled Water
DNA : Deoxyribonucleic acid
DP : Estradiol Decreasing Expression Pattern
E_1 : Estrone
E_2 : Estradiol
E_3 : Estriol
EDTA : Ethylenediaminetetraacetic acid
ER : Estrogen receptor
ESI : Electrospray ionization
EtOH : Ethanol
FAM : Carboxyfluorescein
G-6-P : Glucose-6-Phosphate
HER2 : Human Epidermal Growth Factor Receptor 2
HPLC : High Performance Liquid Chromatography
IBIS : International Breast Cancer Intervention Study
IDC : Invasive Ductal Carcinoma
IHC : Immunohistochemistry
IL-11 : Interleukin-11
IP : Estradiol Increasing Expression Pattern
LC-MS/MS : Liquid Chromatography-Tandem Mass Spectrometry
LOD : Limit Of Detection
LOQ : Limit Of Quantitation
MeOH : Methanol
MGB : Minor Groove Binder
MN : McNemar Test
MRM : Multiple Reaction Monitoring
mRNA : Messenger Ribonucleicacid
MS : Mass Spectrometry
MU : Manwhitney U Test
N : Tumor-Free Breast Tissue Samples

NADPH : Nicotinamide Adenine Dinucleotide Phosphate
P : Tumor Neighboring Breast Adipose Tissues Samples
P450arom : Aromatase Cytochrome P450
P450c17 : Cytochrome P450 17A1 Enzyme
PBS : Phosphate Buffered Saline
PMSF : Phenylmethylsulfonyl Fluoride
PPAR-γ : Peroxisome Proliferator-Activated Receptor Gamma
PR : Progestin Receptor
qRT-PCR : Real-Time Polimerase Chain Reaction
RIA : Radioimmunoassay
RQ : Relative Quantification
SPE : Solid Phase Extraction
StAR : Steroidogenic Acute Regulatory protein
T : Breast Tumor Tissue Samples
TBP : TATA binding box protein
TES : Testosterone
TNF : Tissue Necrosis Factor
UNG : Uracil N-glycosylase
WHO : World Health Organization
WSR : Wilcoxon Signed Ranks test
κ : Kappa

LIST OF TABLES

Page

LIST OF FIGURES

Page

INVESTIGATION OF CYP17A1 AND CYP19A1 GENE EXPRESSION LEVELS AND AROMATASE ACTIVITY IN INVASIVE DUCTAL BREAST CANCER TISSUES

SUMMARY

Breast cancer is the most common cancer in women worldwide, with nearly 1.7 million new cases diagnosed in 2012. It is the second most common cancer overall. This represents about 12% of all new cancer cases and 25% of all cancers in women. The numbers of incidence and mortality is increasing especially in developing countries as well as in Turkey.

Breast cancer is more commonly hormone driven and the factors that modify the risk of this cancer when diagnosed premenopausally and when diagnosed postmenopausally are not the same. Extensive research and clinical observations in the past 20 years confirmed that the cessation of ovarian function at menopause does not stop the process of sex steroid hormone synthesis in females. Currently we acknowledge that multiple extra-ovarian tissues contain the same enzymatic machinery the ovary uses which can maintain a significant rate of local hormonal synthesis sufficient to cause pathological outcomes. This is commonly termed "intracrine". The term intracrinology was first coined over 2 decades ago but there are still questions to be answered, which could help us to understand the intracrine mechanisms in the breast cancer microenvironment. CYP17A1 (P450c17) and CYP19A1 (aromatase) are two of the key enzymes in the central pathways of sex steroid metabolism. In current study, local expressions of CYP17A1, CYP19A1 genes and specific activity levels of aromatase in invasive ductal breast carcinoma tissues were investigated by means of revealing their effect over peripheral and/or intratumoral estrogen production in invasive ductal breast carcinoma tissues. The relationship between these expressions and specific activity status along with the patients' known breast health risk factors and clinicopathological parameters were also reported in order to investigate the effect of tumor progression.

One tumor and one peripheral mammary adipose tissue sample (P) adjacent to the tumor was obtained from each patient (n= 20) and snap frozen in liquid nitrogen and kept at -80°C until use for the extraction of total RNA and microsome isolation. Real-time polymerase chain reaction was employed for the detection of CYP17A1 and CYP19A1 gene expression. All patients were postmenopausal, diagnosed for invasive ductal breast carcinoma and classified as luminal A. Patients were divided into groups according to cilinicopathologic features and breast cancer risk factors. In addition, 12 tumor-free breast tissue samples (N) were obtained from premenopausal women with no history of breast cancer who underwent reduction mammoplasty surgery as the control group. The conversion of testosterone to 17ß-estradiol was determined via radioimmunoassay and liquid chromatography-tandem mass spectrometry, and the specific activity of aromatase in microsomal fractions were calculated. Microsomes were isolated from each specimen by employing differential

centrifugation method for the activity assays. Bicinchoninic acid protein assay was used for detection and quantitation of the total protein in the samples.

The acquired data pointed out the estradiol at the breast tumor microenvironment, which plays a major role in proliferation of malignant epithelial breast cancer cells, was depending on the power of local aromatization activity and the basis of this estrogen drive in the postmenopausal period is the adipose tissue adjacent to the tumor itself. The local aromatase overexpression and high aromatase activity are important factors for the survival of estrogen dependent breast carcinoma cells. Furthermore, there was a pattern consisting the combination upregulated and unaltered gene expressions of CYP17A1 and CYP19A1 which was observed to be coralated with higher aromatase activity levels in peripheral tissues compared to tumor tissues.

To summarize, present study suggesting a complex breast tumor progression mechanism altered by CYP17A1 and CYP19A1 at the breast tumor microenvironment. The evaluation of various clinicopathological and disease risk factors along with the expression levels of CYP17A1 and CYP19A1 and the aromatase activity levels at breast tumor microenvironment might help clinicians to decide on treatment startegies and diagnosis for individual cases, particularly with postmenapausal status. The in-house liquid chromatography-tandem mass spectrometry method has the potential to be further developed to a commonly applied high-throughput technique for aromatase activity measurement which might be an invaluable asset for rapid and specific analysis. However, future studies must be conducted using greater sample size and addition of other key enzyme activities evaluations such as 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase in streidogenesis pathway which effect local estrogen levels for confirmation and gettting more strong and reliable results.

DUKTAL MEME KANSERİ OLGULARINDA CYP17A1 VE CYP19A1 GEN BÖLGELERİNİN EKSPRESYONLARININ VE AROMATAZ AKTİVİTELERİNİN İNCELENMESİ

ÖZET

Meme kanseri tüm dünyada kadınlar arasında en sık görülen kanser türüdür. 2012 yılı verilerine göre yaklaşık 1.7 milyon yeni meme kanseri tanısı konulmuştur. Genel olarak bakıldığında ise en sık rastlanan ikinci kanser türüdür. Bunun anlamı tüm yeni tanı konmuş kanserlerin %12'sini ve tüm kadınlarda görülen kanserlerin %25'ini meme kanseri oluşturmaktadır. Sıklık ve ölüm oranları özellikle Türkiye gibi gelişmekte olan ülkelerde artış göstermektedir.

Meme kanseri için bazı risk faktörleri belirlenmesine karşın tanısı konulan hastaların çoğu için spesifik risk faktörleri tespit etmek mümkün değildir. Yüksek penetrasyon genleri olarak adlandırılan, özellikle BRCA1, BRCA2 ve p53 gibi, bir takım genlerdeki mutasyonların meme kanseri riskini çok yükselttiği bilinmektedir. Ancak, bu mutasyonlara çok sık rastlanmaz ve dolayısıyla mevcut vakaların az bir kısmını açıklamaktadır. Erken menarş, geç menopoz, geç yaşta ilk doğum gibi endojen östrojenlere uzun süre maruz kalma ile ilişkili üreme faktörleri meme kanseri için en önemli risk faktörleri arasında yer almaktadır. Duktal epitel meme hücrelerindeki proliferasyonun uyarılmasının, östrojenlerin karsinogenez üzerindeki ana etkisi olduğu ileri sürülmüştür.

Meme kanseri sıklıkla hormonal kaynaklı olup, bu kanseri modifiye eden risk faktörleri menopoz öncesi ve menopoz sonrası teşhis edildiğinde farklılıklar göstermektedir. Yirmi yılı aşkın süredir süren yoğun çalışmalar ve klinik gözlemler kadınlarda menopoz sonrası yumurtalık fonksiyonlarının kaybedilmesine rağmen cinsiyet steroid hormonlarının sentezlenmesinin devam ettiğini kanıtlamıştır. Günümüzde, yumurtalık dışı bazı dokuların yumurtalıklardakine benzer ve patolojik bir takım sonuçlara sebebiyet verebilecek kapasitede hormon sentezi yapabilecek bir enzimatik sisteme sahip olduğu bilinmektedir. Menopoz sonrası kadınlarda, dolaşımdaki plasma östrojen seviyelerinin düşük olduğu bilinmektedir ancak meme karsinogenezinde gerçekleşen lokal ve intratümoral sentez, tümör dokularında yüksek seviyelerde östrojen görülmesine neden olabilir. Bu durum genel anlamda "intrakrin etki" olarak adlandırılır. İntrakrinoloji teriminin ortaya atılmasının ardından yirmi yılı aşkın bir süre geçmesine karşın halen meme kanseri mikro-çevresindeki intrakrin mekanizmaları anlamak için bize yardımcı olacak bir takım sorulara henüz yanıt bulunamamıştır.

Östrojen biyosentezi yolağı, kolesterolden C19 androjenler ve C18 östrojenlerin sentezine kadar bir seri uzun enzimatik adımlardan oluşur. CYP17A1 (P450c17) ve CYP19A1 (aromataz) bu seks steroidlerinin metabolizmasının merkez yolağında bulunan iki enzimdir. P450c17 ve aromatazın katalizlediği reaksiyonlar bu yolakta hız sınırlayıcı basamakları oluşturduğundan özellikle önemlidirler. C21 steroidlerinin

P450c17 tarafından hidroksilasyonu ve ardından parçalanmasıyla C19 steroidleri androstenedion ve dehidroepiandrosteronlar sentezlenir. Aromataz ise son basamak olan androjenlerden östrojenlerin sentezini katalizler. Mevcut çalışmalardan elde edilen bilgiler CYP17A1 ve CYP19A1 lokal gen ifade seviyelerinin potansiyel prognostik moleküler marker olarak kullanılabileceğini düşündürmektedir. Çalışmamızda invaziv duktal meme kanseri doku örneklerindeki CYP17A1 ve CYP19A1 genlerinin lokal ifadesi ve aromatazın spesifik aktivitesi incelenmiştir. Böylelikle bu parametrelerin tümör dokusunun kendi içerisindeki ve/veya çevresindeki östrojen üretimini nasıl etkilediğini değerlendirmek mümkün olmuştur. Gen ifadesi ve aromataz aktivite seviyelerinin yanı sıra hastalara ait bilinen meme sağlığı risk faktörleri ve klinikopatalojik parametreler dikkate alınarak tümör gelişimine olan etkileri incelenmiştir. Ayrıca klinik anlamda bakıldığında, östrojen bağımlı meme kanseri vakalarında aromataz aktivite seviyelerini hassas, doğru ve hızlı bir şekilde ölçen bir yönteme ihtiyaç olduğu görülmektedir. Bu amaç doğrultusunda meme dokusundan spesifik aromataz aktivite ölçümlerinin gerçekleştirilebileceği radyoimmün test ve likit kromatografi-sıralı kütle spektrometresi yöntemleri geliştirilmiştir.

Her bir hastaya (n= 20) ait bir tümör (T) ve bir çevre adipoz (P) meme doku örneği toplanmıştır. Alınır alınmaz sıvı azot ile dondurulan doku örnekleri RNA ve mikrozom izolasyonu yapılana kadar -80°C'de muhafaza edilmiştir. CYP17A1 ve CYP19A1 genlerinin dokulardaki ifade düzeyleri gerçek zamanlı polimeraz zincir reaksiyonu yöntemi ile incelenmiştir. Tüm hastalar menopoz sonrası durumunda olup, duktal invaziv meme kanseri tanısı konmuş ve sınıflandırma açısından luminal A tipine dahil olan vakalarıdır. Hastalar klinikopatolojik parametrelere ve taşıdıkları meme kanserine yakalanma risk faktörlerine göre gruplandırılmışlardır. Bunlara ek olarak meme küçültme ameliyatı olan ve herhangi bir kanser geçmişi olmadığı bilinen, menopoz öncesi durumdaki 12 hastanın rezeksiyon materyallerinden, kontrol grubu olarak kullanılmak üzere meme adipoz doku örnekleri (N) alınmıştır. Testosteronun 17ß-estradiole dönüşümü radyoimmün test ve likit kromatografi-sıralı kütle spektrometresi yöntemleri kullanılarak tespit edilerek mikrosomal fraksiyonlardaki spesifik aromataz aktivitesi hesaplanmıştır. Mikrozomal fraksiyon her bir örnekten diferansiyel santrifüjleme ile elde edilmiştir. Örneklerdeki toplam protein miktarı bikinkoninik asit protein analiz yöntemi ile tespit edilmiştir. Gruplar arası mRNA seviyelerindeki ve spesifik aromataz aktivitelerindeki anlamlı farklılıkların belirlenmesinde uygunluğuna göre Wilcoxon, Mann-Whitney U ve McNemar testleri gibi non-parametrik testler ile analizler gerçekleştirilmiştir.

Elde edilen sonuçlar meme tümörü mikro-çevresinde gerçekleşen ve kötü huylu meme kanseri epitel hücrelerinin proliferasyonunda temel bir rol üstlenen estradiol biyosentezinin, CYP17A1 ve CYP19A1 gen ifadeleri ve lokal aromatizasyon aktivitesi tarafından etkilendiğini göstermektedir. Doku tiplerine göre bakıldığında CYP17A1 gen ifadesi seviyeleri sağlıklı bireylerdeki meme dokusunda (N) en yüksek olmak üzere, ardından tümörün çevresinde yer alan doku (P) ve tümör dokusunun kendisi (T) şeklinde sıralanmaktadır. CYP19A1 gen ifadesi seviyesi ise çevre dokularda diğer doku gruplarına göre oldukça yüksek bulunmuştur. Tüm vakalara bakıldığında, çevre dokularda tümöre göre CYP19A1'in kuvvetli bir şekilde upregüle olduğu ($p=0.001$) buna karşın CYP17A1'de ise hafif bir upregülasyon ($p=0.687$) olduğu gözlenmiştir. Bulgular menapoz sonrası dönemde estrojen kaynağının tümörün yakın çevresinde yer alan fibroblast ve preadipozit hücreler

olduğu hipotezini destekler niteliktedir. Mevcut literatür bilgisi dikkate alınarak iki gen birlikte ifade düzeylerine göre lokal estrojen sentezini arttırıcı (IP) ve azaltıcı (DP) olarak gruplanarak analiz edilmiştir. CYP17A1 ve CYP19A1'in upregüle ve değişmemiş olduğu durumların birleşiminden oluşan arttırıcı grubun, çevre dokularda görülen yüksek aromataz aktivitesi ile korelasyona sahip olduğu tespit edilmiştir.

Çevre doku ve tümör doku arasındaki gen ifade seviyelerini farkının birçok hasta karakteristiği tarafından etkilendiği görülmüştür.

Özetle, bu çalışma meme kanseri mikroçevresindeki CYP17A1 ve CYP19A1 gen ifadesi değişimlerinin, karmaşık bir mekanizma üzerinden meme kanseri gelişimini etkilediği göstermektedir. Bahsedilen genlerin ifadesi ile birlikte meme tümörü mikroçevresindeki aromataz aktivitesinin çeşitli klinikopatolojik bulgular ve hastalık risk faktörleri de dikkate alınarak incelenmesinin, klinisyenlere kişiye göre, özelikle menapoz sonrası hastalarda, teşhis ve tedavi stratejilerini belirlemede yardımcı olacağı düşünülmektedir. Çalışma sonucu geliştirilmiş olan likit kromatografi-sıralı kütle spektrometresi yöntemi hızlı ve spesifik aromataz aktivite ölçümü için rutinde kullanılabilecek yüksek çıktılı bir analiz olma potansiyeli yüksektir. Ancak daha etkin ve güvenilir sonuçlar elde etmek adına, steroidogenez yolağındaki bölgesel östrojen sentezini etkileyebilecek 3β-hidroksisteroid dehidrogenaz ve 17β-hidroksisteroid dehidrogenaz gibi diğer önemli enzimlerin de aktivite değişimlerini değerlendirerek, daha büyük bir örneklem boyutu ile çalışmalar düzenlemek faydalı olacaktır.

1. INTRODUCTION

Every year almost two-milion women worldwide were told, “You have breast cancer”. Breast cancer is globally the most common form of cancer in women. Current statistics shows that one in eight women is at risk for developing breast cancer during their life time (Ferlay et al, 2015).

The causes of breast cancer are still not fully known. For the past three decades breast cancer risk factors have been studied, and the single most important risk factor, except gender, seems to be age. Nearly half of the women, who have breast cancers, have no other risk factors except age and gender. The risk of breast cancer increases among women older than 50 years of age who have benign breast disease, especially those with atypical ductal or lobular hyperplasia. Both lobular and ductal carcinoma in situ increases risk significantly, as do a family history of breast cancer in first-degree relatives and the presence of BRCA1 or BRCA2 mutations. Diet, exercise, and environmental factors play a very small role in overall risk. On the other hand mammographic breast density increases relative risk fivefold among women with the highest density, and breast cancer risk is two to three times greater in women with elevated serum levels of estradiol or testosterone. Hormonally linked adult reproductive and anthropometric risk factors, such as young age at menarche (<12 years), older age at first birth (>30 years), null parity and older at the age of menopause (>55 years,) may contribute to the etiology of postmenopausal breast cancer (Vogel, 2008).

All these can be considered as breast cancer risk increasers and have to deal with the fact that these women have exposed to more estrogen throughout their lives. As we know breast cancers are often hormonally driven and estrogen receptor positive (ER (+)). About 70 - 80% of all newly diagnosed breast cancers are positive for the estrogen receptor and some degree positive for progesterone receptor (Hammond et al, 2010). All of these information points out that estrogen has a key role as a promoter of tumor growth.

The effect of estrogens in breast carcinogenesis has been investigated in cell culture, animal models, and humans. Breast tissue is becoming the focus point as an intracrine organ, with potentially important local estrogen production (Yaghjyan and Colditz, 2011).

Aromatase (CYP19A1) and Cytochrome P450 17A1 (CYP17A1) are two of the key enzymes involved in estrogen biosynthesis (Figure 1.1).

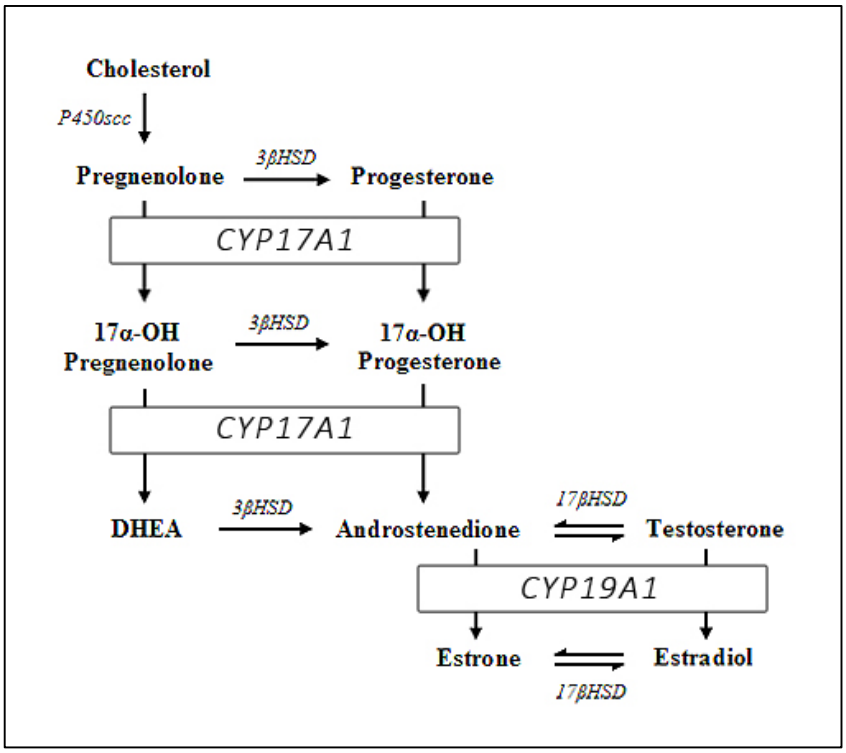

Figure 1.1: The role of CYP17A1 and CYP19A1 in estrogen biosynthesis pathway.

Enhancement of aromatase expression and activity have been shown in various cancers, including breast tumors, hepatocellular carcinoma, adrenocortical tumors and testicular tumors (Bulun and Simpson, 2008; Jongen et al, 2006; Carruba, 2009; Bulun et al, 1997; Young et al, 1996; Aiginger et al, 1981). Particularly in the case of breast cancer, it is strongly possible that local paracrine and/or intracrine estrogen signaling is stimulating the progression and recurrence of the disease, especially in hormone receptor positive carcinomas. Paracrine interactions between malignant breast epithelial cells, proximal adipose fibroblasts, and vascular endothelial cells are responsible for estrogen biosynthesis and the lack of adipogenic differentiation in breast cancer tissue. It is most likely malignant epithelial cells secrete factors that inhibit the differentiation of surrounding adipose fibroblasts for their maturation and

stimulate aromatase expression in these undifferentiated adipose fibroblasts (Meng et al, 2001). The in vivo presence of malignant epithelial cells also enhances aromatase expression in endothelial cells in breast tissue (Zhou et al, 2001). After ovarian function subsides during menopause or after another pathological change or medical intervention that reduces or eliminates ovarian function, peripheral estrogen synthesis by aromatase becomes the primary pathway for the production of estrogen in women. In addition to Aromatase, CYP17A1 activity, which is at a critical crossroad point in the pathways of steroid hormone biosynthesis, also has been demonstrated in breast cancer tissues more than three decades ago (Abul-Hajj et al, 1979). The relation between breast cancer and CYP17A1 have been considerably evaluated but findings are mixed and no firm conclusions can be drawn at present (Feigelson et al, 1997; Helzlsouer et al, 1998; Setiawan et al; 2007; Chen et al, 2008). Although studies suggest a possibility that CYP17A1 may be involved in in situ synthesis of estrogens, and the overexpressions of CYP17A1 messenger ribonucleicacid (mRNA) might affect intratumoral estrogen levels as well.

1.1 Breast Cancer by Numbers in The World and Turkey

Breast cancer, with an estimated number of 1.67 million new cases in 2012, is by far the most common cancer diagnosed in women worldwide (ranking second when both sexes combined). It means that nearly a quarter of all cancers diagnosed in women is breast cancer (25%) (Ferlay et al, 2015). In most countries worldwide, incidence of breast cancer has increased in the last decades, with the most rapid increases occurring in developing countries underlying causes are thought to be the differences in reproductive behavior, the use of exogenous hormones, as well as differences in weight, exercise, diet and alcohol consumption among these countries (Beral and Million Women Study Collaborators, 2003; Reeves et al, 2007; Monninkhof et al, 2007; Allen et al, 2009).

Across the regions of the world, female breast cancer incidence rates vary nearly five-fold. The highest incidence rates in 2012 belong to Belgium and Denmark (111.9 and 105 age standardized rate per 100000, respectively). Figure 1.2 is showing the 2012 estimates of the incidence rates in Western Asia region which Turkey is included. The average incidence rate is 42.8 per 100000. With the number of 39.1, Turkey is in 13th place across the region (Ferlay et al, 2015).

The most common cause of death from cancer in women worldwide is also breast cancer (ranking fifth when both sexes combined). It's estimated to be responsible for almost 522,000 deaths in 2012. Its nearly one third of the newly diagnosed cases. Variation in female breast cancer mortality across the regions of the world is less, largely due to better survival in the (high incidence) developed countries.

According to GLOBOCAN 2012 data, the average mortality rate of Western Asia region is 15.1 per 100000. Turkey is in 11th place across the region with 13.4 per 100000 (Ferlay et al, 2015).

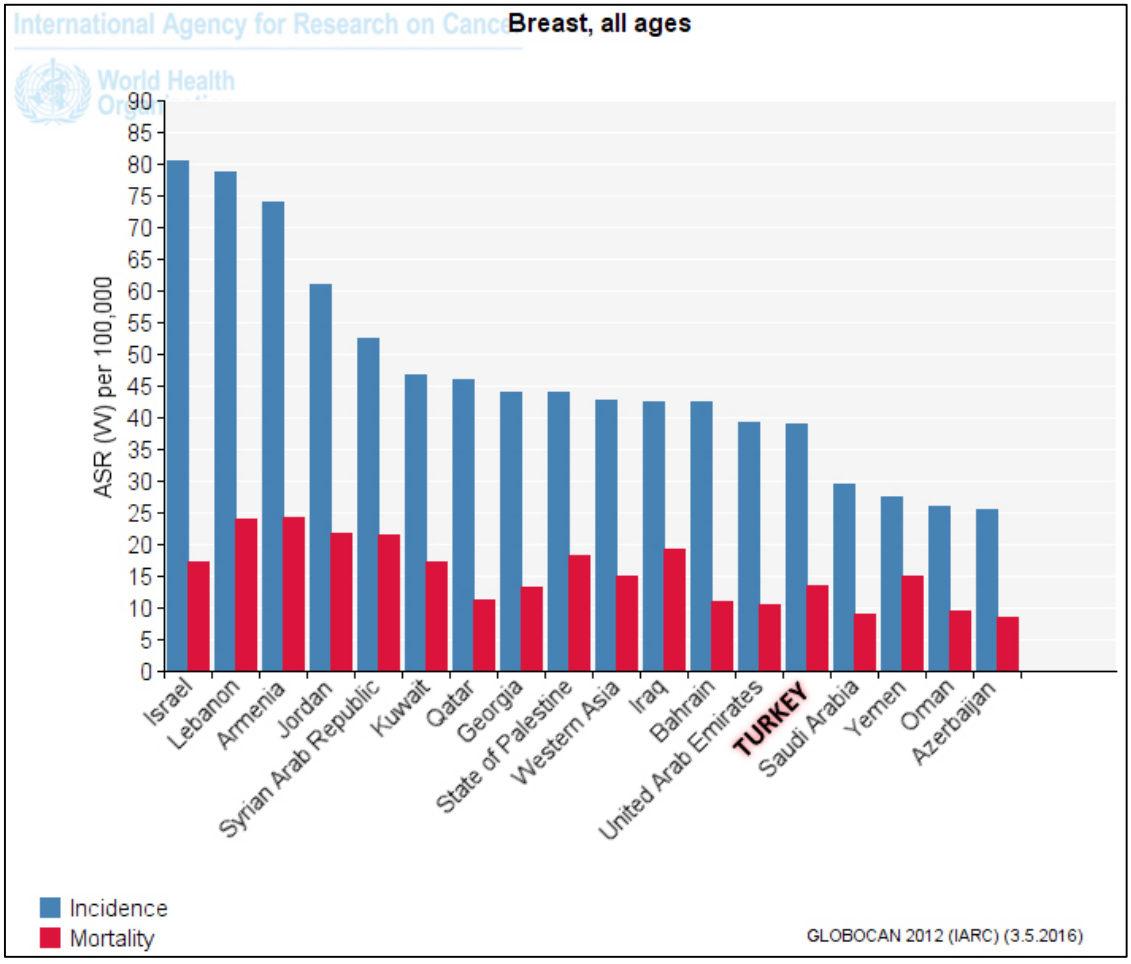

Figure 1.2: Breast cancer, females, Western Asia age-standardised incidence and mortality rates, 2012 estimates.

According to data of the Department of Cancer Control, incidence rate for Turkish women was 38.6 per 100000 in 2010 (Figure 1.3) (Köse et al, 2014).

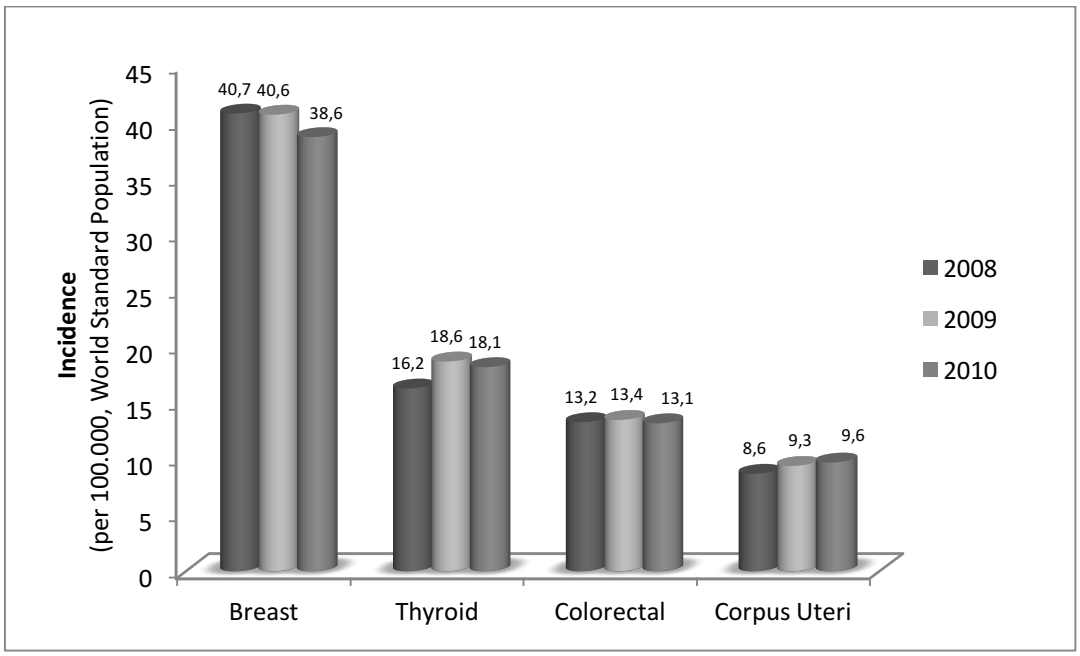

Figure 1.3: Incidence of the most common four types of cancer in women in Turkey, 2008-2010 (per 100.000, World standard population).

If we look at the future prospects, World Health Organization (WHO) estimates are suggesting 64% and 49% increase in incidence and mortality numbers in Turkey between 2015 and 2035 (Figure 1.4).

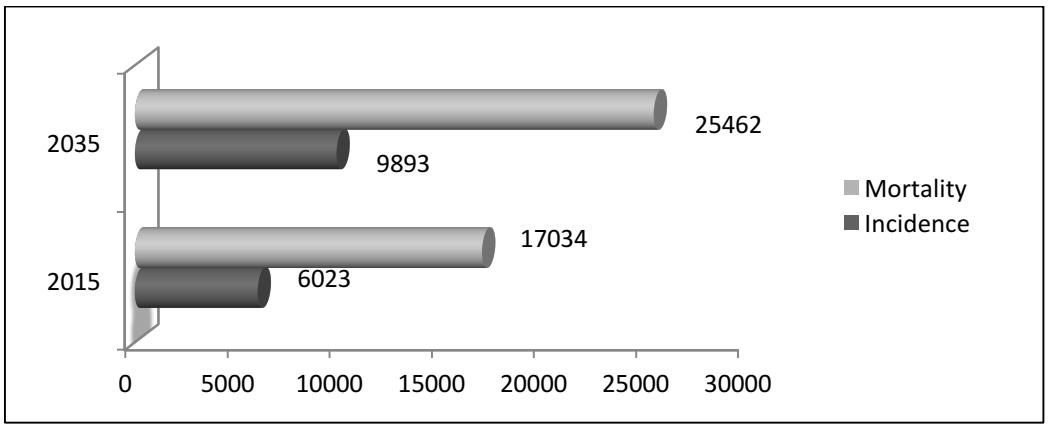

Figure 1.4: Estimated number of new breast cancer cases and mortality in Turkey.

Although in the last decade, incidence rate and prevalance have increased three times in Turkey, there is not a nationwide screening program yet. There are big differences regarding stage at diagnosis, and effective treatments between eastern and western part of Turkey which is largely due to late presentation of the disease, limited resources for diagnosis and treatment, lack of breast health awareness, social, cultural, and educational factors. The most fundamental interventions are early

detection, diagnosis, surgery, radiation therapy, and drug therapy can be integrated and organized within existing health care schemes in Turkey and other low and middle income countries (Özmen et al, 2009).

1.2 Classification of Breast Cancer

Breast cancer is a cancer that starts in the breast, usually in the inner lining of ducts or lobules. Although the definition seems to be simple, classification of the disease can be complex. Breast cancers can be classified according to different aspects. Each of these can influence treatment response and prognosis. Description of a breast cancer would optimally include all of these classification aspects, as well as other findings, such as signs found on physical exam. A full classification includes histopathological type, grade, stage (TNM), receptor status, and the presence or absence of genes as determined by genetic testing.

1.2.1 Histopathologic classification

According to the World Health Organization (WHO), there are more than 100 types and subtypes of breast tumors. Simplified version of this classification is shown in Table 1.1 (Lakhani et al, 2012).

Table 1.1: Simplified classification of tumors of the breast.

Epithelial tumours		Others
Invasive ductal carcinoma	Lobular neoplasia	Myoepithelial lesions
Invasive lobular carcinoma	Intraductal proliferative lesions	Mesenchymal lesions
Tubular	Microinvasive ca	Fibroepithelial tumours
Invasive cribriform	Intraductal papillary neoplasms	Tumours of the nipple
Medullary	Benign epithelial proliferations	Malignant lymphoma
Mucinous ca and other tumours with abundant mucin	Adenomas	Metastatic tumours
Neuroendocrine tumors	Oncocytic ca	Tumours of the male breast
Invasive micropapillary ca	Adenoid cystic ca	
Apocrine ca	Acinic cell ca	
Metaplastic carcinomas	Glycogen-rich clear cell ca	
Lipid-rich ca	Sebaceous ca	
Secretory ca	Inflammatory ca	
Oncocytic ca	Invasive papillary ca	
Adenoid cystic ca		
Acinic cell ca		

Ductal carcinomas are members of epithelial tumors in this classification. Invasive ductal carcinoma (IDC), which is our study group belongs, is the most common type

of breast cancer. It refers that cancer cells has broken through the wall of the milk duct and begun to invade the tissues of the breast (Figure 1.5).

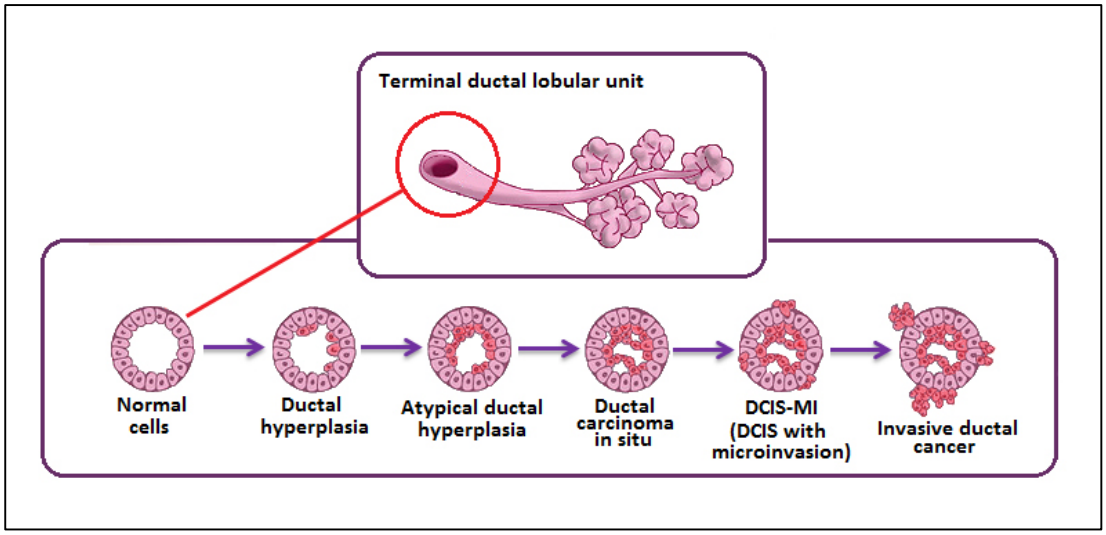

Figure 1.5: Invasive ductal breast cancer and the progression model.

1.2.2 Grading

The Nottingham (also called Elston-Ellis) modification of the Scarff-Bloom-Richardson grading system, is recommended, which grades breast carcinomas by adding up scores for tubule formation, nuclear pleomorphism, and mitotic count, each of which is given 1 to 3 points (Elston and Ellis, 2002; Bloom and Richardson, 1957; Genestie et al, 1998). The scores for each of these three criteria and then added together to give an overall final score and corresponding grade as follows:

- 3-5 Grade 1 tumor (well-differentiated). Best prognosis.
- 6-7 Grade 2 tumor (moderately-differentiated). Medium prognosis.
- 8-9 Grade 3 tumor (poorly-differentiated). Worst prognosis.

Lower grade tumors, with a more favorable prognosis, can be treated less aggressively, and have a better survival rate. Higher grade tumors are treated more aggressively, and their intrinsically worse survival rate may warrant the adverse effects of more aggressive medications.

1.2.3 Staging

The TNM classification for staging breast cancer is based on the size of the cancer where it originally started in the body and the locations to which it has moved. These

cancer characteristics are described as the size of the tumor (T), whether or not the tumor has spread to the lymph nodes (N) in the armpits, neck, and inside the chest, and whether the tumor has metastasized (M) (Table 1.2) (Edge et al 2010). Larger size, nodal spread, and metastasis have a larger stage number and a worse prognosis. Stage 0 which is in situ disease or Paget's disease of the nipple. Stage 0 is a pre-cancerous or marker condition, either ductal carcinoma insitu (DCIS) or lobular carcinoma insitu (LCIS). Stages I–III are within the breast or regional lymph nodes. Stage IV is a metastatic cancer. Metastatic breast cancer has a less favorable prognosis.

Table 1.2: Anatomic stage/prognostic groups.

Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T0	N1mi	M0
	T1*	N1mi	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1*	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

1.2.4 Molecular Subtype

The receptor status of breast cancers has traditionally been identified by immunohistochemistry (IHC), which stains the cells based on the presence of Estrogen Receptors (ER), Progestin Receptors (PR) and Human Epidermal Growth Factor Receptor 2 (HER2). This remains the commonest method of testing for receptor status, but deoxyribonucleic acid (DNA) multi-gene expression profiles can categorize breast cancers into molecular subtypes that generally correspond to IHC receptor status (Table 1.3).

Table 1.3: Molecular subtypes of breast tumors (Yang et al; 2011).

Subtype	These tumors tend to be
Luminal A	ER (+) and/or PR (+), HER2-, low Ki67
Luminal B	ER (+) and/or PR (+), HER2 (+) (or HER2 (-) with high Ki67)
Triple negative/basal-like	ER (-), PR (-), HER2 (-), cytokeratin 5/6 (+) and/or HER1 (+)
HER2 (+)	ER (-), PR (-), HER2 (+)

Receptor status is a critical assessment for all breast cancers as it determines the suitability of using targeted treatments such as tamoxifen and or trastuzumab. These treatments are now some of the most effective adjuvant treatments of breast cancer. ER+ cancer cells depend on estrogen for their growth, so they can be treated with drugs to reduce either the effect of estrogen (e.g. tamoxifen) or the actual level of estrogen (e.g. aromatase inhibitors), and generally have a better prognosis. Generally, prior to modern treatments, HER2 (+) had a worse prognosis (Sotirou and Pusztai, 2009), however HER2 (+) cancer cells respond to drugs such as the monoclonal antibody, trastuzumab, (in combination with conventional chemotherapy) and this has improved the prognosis significantly (Romond et al., 2005). Conversely, triple negative cancer (i.e. no positive receptors), lacking targeted treatments now has a comparatively poor prognosis (Dent et al, 2007).

Androgen receptor is expressed in 80-90% of ER (+) breast cancers and 40% of "triple negative" breast cancers. Activation of androgen receptors appears to suppress breast cancer growth in ER (+) cancer while in ER (-) breast it appears to act as growth promoter. Efforts are underway to utilize this as prognostic marker and treatment (Lehmann et al, 2011; Hu et al, 2011).

Receptor status was traditionally considered by reviewing each individual receptor (ER, PR, HER2) in turn, but newer approaches look at these together, along with the tumor grade, to categorize breast cancer into several conceptual molecular classes that have different prognoses and may have different responses to specific therapies (Prat and Perou, 2011; Geyer et al, 2009).

1.2.5 Other classification approaches

Understanding the specific details of a particular breast cancer may include looking at the cancer cell DNA by several different laboratory approaches. When specific

DNA mutations or gene expression profiles are identified in the cancer cells this may guide the selection of treatments, either by targeting these changes, or by predicting from the DNA profile which non-targeted therapies are most effective.

1.3 Estrogens and Breast Cancer

The growth of female breast depends upon several hormones, the most important of which is estrogen. Estrogen governs the development of the ductal system of the breast whereas progesterone is responsible for the proper development of the lobular system.

There are three major estrogens naturally synthesized in women; estrone (E_1), estradiol (E_2), and estriol (E_3). The predominant estrogen during reproductive years both in terms of absolute serum levels as well as in terms of estrogenic activity is E_2. The biologically active estrogen E_2 is produced in at least three major sites: 1) direct secretion from the ovary in reproductive-age women; 2) by conversion of circulating androstenedione (A) of adrenal and/or ovarian origins to E_1 in peripheral tissues; and 3) by conversion of A to E_1 in estrogen-target tissues (Figure 1.6). During menopause, estrone is the predominant circulating estrogen and during pregnancy estriol is the predominant circulating estrogen in terms of serum levels. Though estriol is the most plentiful of the three estrogens it is also the weakest, whereas estradiol is the strongest. Thus, estradiol is the most important estrogen in non-pregnant females who are between the menarche and menopause stages of life. All of the different forms of estrogen are synthesized from androgens, specifically testosterone and androstenedione, by the enzyme aromatase.

As mentioned in introduction section, lifetime exposure to estrogens correlates with the incidence of breast cancer in women at risk (Santen, 2007). In the hormone-dependent subtype of breast cancers, the role of estrogens as modulators of mitogenesis overrides the influence of other factors. These sex steroids stimulate cell

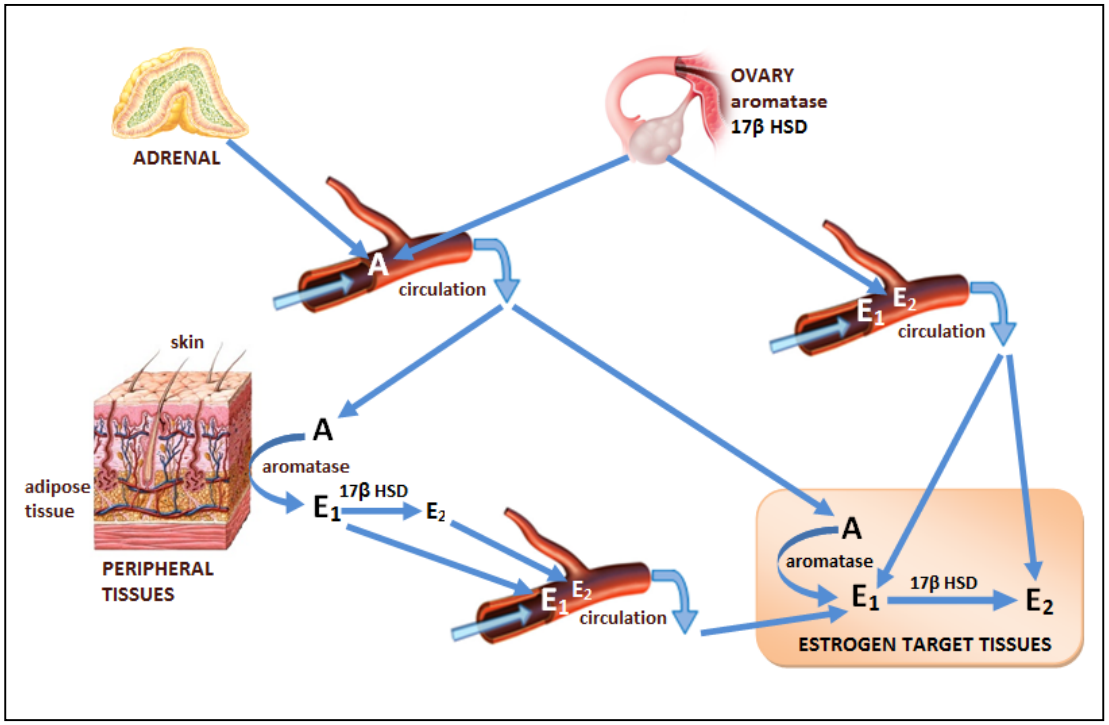

Figure 1.6: Main sources of estrogens in women.

proliferation directly by increasing the rate of transcription of early response genes such as c-myc and indirectly through stimulation of growth factors which are produced largely in response to estrogenic regulation. Enhanced cell proliferation, induced either by endogenous or by exogenous estrogens, increases the number of cell divisions and, by inference, the proportionate number of mutations. With an enhanced rate of proliferation, the time available for DNA repair is reduced. In addition, occurrence of single-stranded DNA, during cell cycle, is more susceptible to damage than double-stranded DNA (Figure 1.7). This is the predominant theory at the present time relates to effects of estrogen on cell growth. Another current theory is that estrogens can be metabolized to genotoxic products. These two current theories of enhanced cell proliferation and genotoxic metabolites are not mutually exclusive but could act in an additive or even synergistic fashion. For example, DNA damage originating from catechol estrogens would be propagated more rapidly by increased cellular proliferation, and insufficient time might be available for DNA repair (Jefcoate et al, 2000).

The risk of breast cancer and exposure to estrogen have a close relation so it is important to examine the key variables in estrogen homeostasis (i.e., the synthesis and catabolism of estrogen and the sensitivity of tissue to estrogen). The initial entry

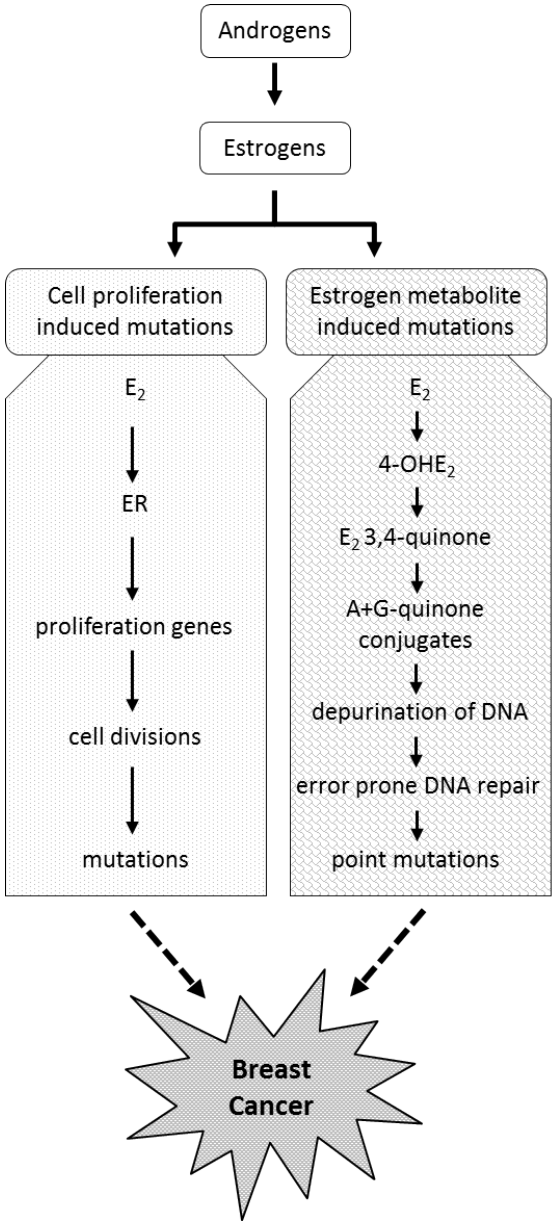

Figure 1.7: Diagrammatic representation of the two pathways whereby estrogens can cause breast cancer.

of cytosolic cholesterol into the mitochondrion, which is facilitated by steroidogenic acute regulatory protein (StAR), represents a major step for steroidogenesis. Six enzymes encoded by at least five specific genes then catalyze the conversion of cholesterol to the biologically active estrogen estradiol. Both CYP17A1 (encoding P450c17) and CYP19A1 (encoding aromatase) are involved in estrogen biosynthesis. In situ aromatization in breast tumors results in increased estrogen in breast tissue, which may contribute to the growth of breast tumors in an autocrine or paracrine manner. Suppression of tissue-specific inhibitors of the promoter may also result in

increased synthesis of aromatase mRNA. Thus, the aromatase gene may act as an oncogene that initiates tumor formation in breast tissue (Clemons and Goss, 2001).

1.4 CYP17A1 and CYP19A1

1.4.1 CYP17A1 and P450c17

The CYP17A1 gene is present in the genomes of all Chordata species and encodes an evolutionarily conserved P450 protein. The human gene spans approximately 10 kb on chromosome 10q24.3, encompassing eight exons separated by seven introns (Picardo-Leonard and Miller, 1987) (Figure 1.8). The human mRNA appears to be ubiquitously expressed in all tissues, with the highest levels detected in testis and adrenals. Transcription is initiated approximately 180-bp upstream of the initiation codon in exon 1 and produces ~1.7 kb mRNA and it encodes a 508 amino acid enzyme (P450c17, EC 1.14.99.9). This enzyme is a membrane-bound dual-function monooxygenase with a critical crossroad point in the pathways of human steroid hormone biosynthesis, catalyzing two different enzymatic reactions, the 17α-hydroxylation and 17,20-lyase reactions of the C21-steroids. 17α-hydroxylase activity is required for generation of glucocorticoids like cortisol, while its hydroxylase and 17,20-lyase activities are required for production of androgenic and estrogenic sex steroids (Voutilainen and Miller, 1986). P450c17 determines the final products in steroid hormone biosynthesis and plays an important role in cell homeostasis. Thus, when the 17α-hydroxylase activity of P450c17 predominates, the biosynthesis of steroid hormones is directed mainly to the biosynthesis of glucocorticoids. If the 17,20-lyase activity of P450c17 is predominant, then biosynthesis of steroid hormones is directed to the production of sex hormones (Martucci and Fishman, 1993).

There are several CYP17A1 polymorphism studies, which are related to breast cancer risk (Kaufman et al, 2011; Wang et al, 2009; Tüzüner et al, 2010). However, there are currently no studies directly investigates the relationship between local gene expressions levels of CYP17A1 gene and breast cancer susceptibility.

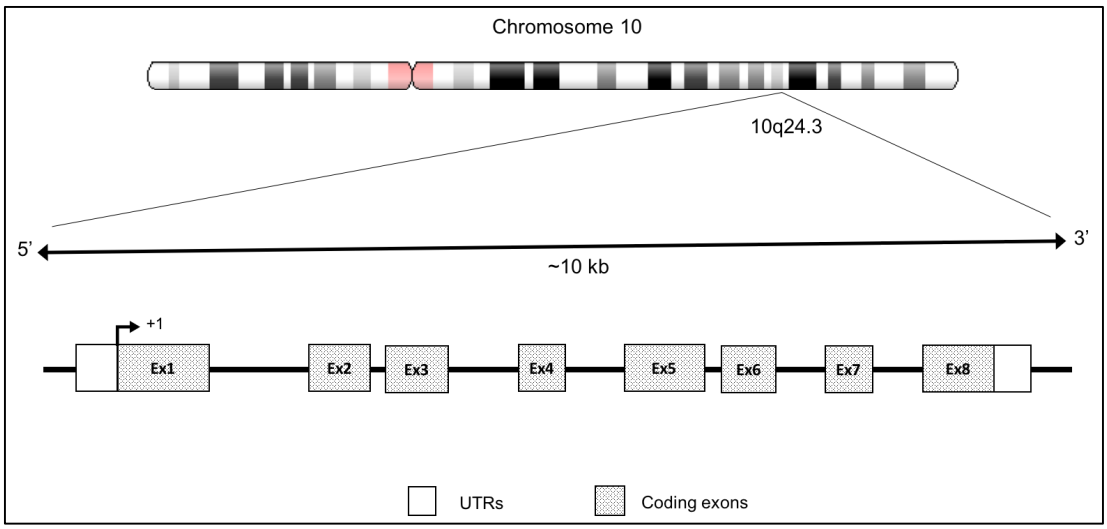

Figure 1.8: Schematic representation of the human CYP17A1 gene.

CYP17A1 is a target for inhibiting the growth of hormone-dependent cancers including breast cancer. Abiraterone, which is a potent and selective CYP17A1 inhibitor, has been proposed as a viable treatment option in women with metastatic ER (+) breast cancer as well as with triple-negative disease that are AR (+). There are still further studies required to better characterize which breast cancer patients may benefit the most (Capper et al, 2016).

1.4.2 CYP19A1 and aromatase

Aromatase is encoded by a single copy of the CYP19A1 gene located on the short arm of chromosome 15 (15q21.2) (Figure 1.9) (Harada et al, 1990). It is approximately 120 kb long and comprises 10 exons. Nine coding exons (II-X) span approximately 30kb, and there are a number of alternative non-coding first exons which are expressed in a tissue- specific manner. As various tissues utilize their own promoters and associated enhancers and suppressors, the tissue-specific regulation of estrogen synthesis is very complex. Although the transcripts for aromatase have different 5' ends in various tissues depending on the promoter usage, these unique first exons are spliced into a common 3'-junction upstream of the start of translation, resulting in the synthesis of identical aromatase proteins (Sebastian and Bulun, 2001).

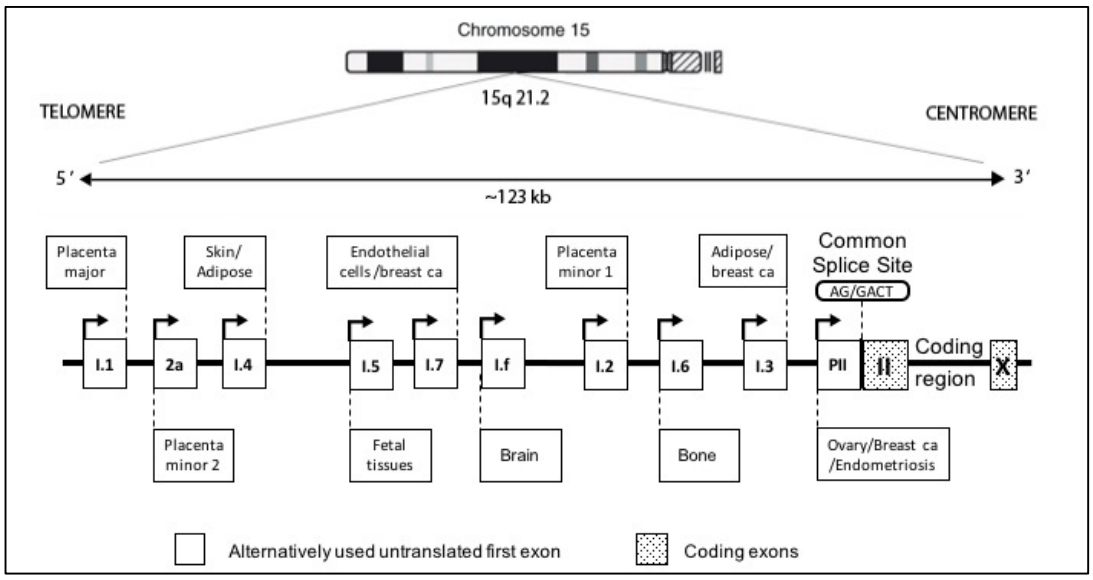

Figure 1.9: Structure of the human CYP19A1 gene. Expression of the aromatase gene is regulated by the tissue-specific activation of a number of promoters via alternative splicing.

The most proximal gonadspecific promoter II and two other proximal promoters, I.3 (expressed in adipose tissue and breast cancer) and I.6 (expressed in bone) are found to be located within the 1 kb region upstream of the ATG translation start site in exon II. Promoter I.2, the minor placenta-specific promoter, is located approximately 13 kb upstream of the ATG site in exon II. The promoters specific for the brain (I.f), endothelial cells (I.7), fetal tissues (I.5), adipose tissue (I.4) and placenta (2a and I.1) are localized in tandem order at ~ 3, 36, 43, 73, 78 and 93 kb, respectively, upstream of the first coding exon, the exon II (Figure 1.9). In addition to promoter II specific sequences, transcripts containing two other unique sequences, untranslated exons I.3 and I.4, are present in adipose tissue and in adipose tissue fibroblasts maintained in culture. Transcription initiated by use of each promoter gives rise to a transcript with a unique 5'-untranslated end that contains the sequence encoded in the first exon immediately downstream of this particular promoter (Figure 1.9). Therefore, the 5'-untranslated region of aromatase mRNA is promoter specific and may be viewed as a signature of the particular promoter used. It should be emphasized again that all of these 5'-ends are spliced onto a common junction 38 bp upstream of the ATG translation start site. Thus, use of alternative promoters does not affect protein structure but its expression level.

1.4.2.1 Aromatase enzyme

Human aromatase is a 58 kDa protein that was purified from placental microsomes in the late 1980s (Mendelson et al, 1987). The protein is highly conserved among all vertebrates. Aromatase enzyme complex is comprised of two polypeptides. The first of these is aromatase cytochrome P450 (P450arom) which is a specific cytochrome P450 and the product of the CYP19A1 gene. The second is a flavoprotein, nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase and is ubiquitously distributed in most cells. Thus, cell-specific expression of P450arom determines the presence or absence of aromatase activity. Since only a single gene (CYP19A1) encodes aromatase in humans, targeted disruption of this gene or inhibition of its product effectively eliminates estrogen biosynthesis (Simpson et al, 2002).

The functional human enzyme is comprised of a heme group and a polypeptide chain of 503 amino acid residues. It is an integral membrane protein of the endoplasmic reticulum, anchored to the membrane by an amino (N)-terminal transmembrane domain (Figure 1.10-A) (Sohl and Guengerich, 2010; Sebastian and Bulun, 2001). Aromatase has been the topic of intense biochemical and biophysical investigations for the past 50 years because of its unique hydroxylation reaction that involves a carbon-carbon bond cleavage and a ring aromatization in the estrogen biosynthesis pathway (Santen et al, 2009; Simpson et al, 2005; Ryan,1959).

Aromatase is the final enzyme in the central pathways of sex steroid metabolism and is probably the best characterised enzyme in intratumoral steroid production in breast cancer. Aromatase irreversibly commits steroids to an estrogenic lineage through aromatisation of the A ring of the steroid backbone in a sequential, three-step reaction (Figure 1.10-C). Aromatase can act on either androstenedione or testosterone (TES), forming either the relatively weaker estrogen–E_1, or the more potent estrogen–E_2. This formation, first identified in 1959 (Ryan, 1959), requires three molecules each of NADPH and O_2 and proceeds through two relatively stable intermediates, the 19-hydroxy and 19-aldehyde compounds, before the final aromatization step. There has been considerable debate over the chemistry of the third step, and two mechanisms are currently favored. The model proposes that the ferric peroxide form of the P450 ($FeOO^-$, Figure 1.10-B) attacks the aldehyde, followed by heterolytic cleavage of the peroxide bond and the transfer of the 1β

proton of the steroid to the heme to generate a ferrous hydroxy intermediate, with the loss of formic acid (Akthar et al, 1982; Cole and Robinson, 1991).

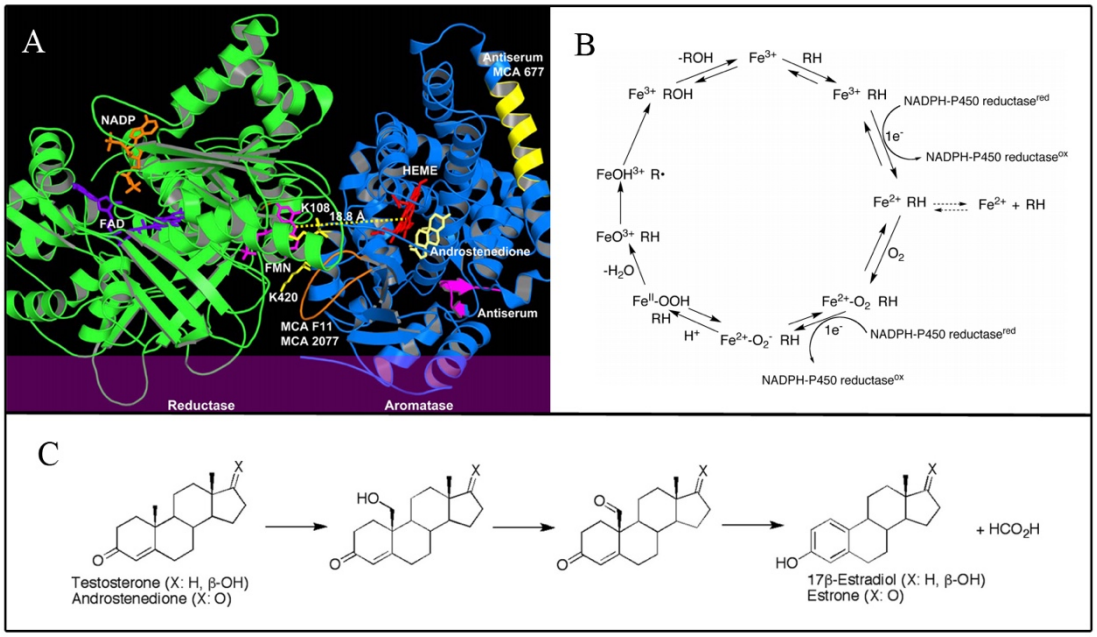

Figure 1.10: The aromatase enzyme complex and conversion of androgens to estrogens. A) Computer-assisted docking model of the aromatase-reductase complex. A ribbon representation of the reductase (green) - aromatase (blue) complex showing its association with endoplasmic reticulum membrane (purple). B) General P450 catalytic cycle. C) The three steps of the A ring aromatization.

It's known that aromatase is expressed by different cell types such as granulosa cells, Leydig and Sertoli cells, placental cells, neurons, preadipocytes and fibroblasts, vasculature smooth muscle cells, chondrocytes, and osteoblasts (Simpson et al, 1994). Therefore, estrogens are not only produced in gonads but also brain, adipose tissue, breast, skin, blood vessels, bone, and cartilage (Simpson, 2003). Expression levels show interpersonal and regional differences, and they are different at various stages of life, e.g. fetal liver expresses aromatase, but it is not present in adult liver (Simpson et al, 2002).

1.4.2.2 Aromatase and breast cancer

The desmoplastic reaction (Figure 1.11) is essential for structural and biochemical support for tumor growth. The "scirrhous cancer" term was used by pathologists for most of the invasive ductal carcinoma cases, indicating the rock-like consistency of these tumors (Haagensen, 1986). Accumulation of fibroblasts around malignant epithelial cells serves to maintain the strikingly hard consistency in many of these

tumors and increased local concentrations of estrogen via aromatase overexpression localized to these undifferentiated fibroblasts.

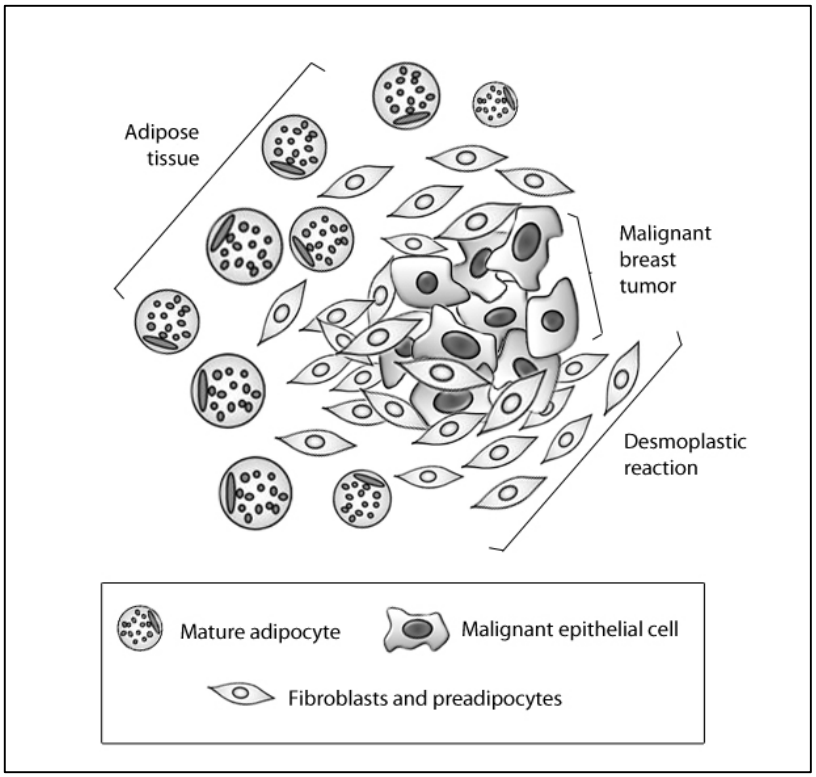

Figure 1.11: The desmoplastic tissue reaction in breast cancer.

Extraordinarily large quantities of tissue necrosis factor (TNF) and interleukin-11 (IL-11) are produced and secreted by malignant breast epithelial cells (Meng et al, 2001). Therefore, large numbers of these estrogen-producing cells are maintained adjacent to malignant cells. At the same time, a separate set of factors secreted by malignant epithelial cells activates aromatase expression in surrounding adipose fibroblasts (Meng et al, 2001). This tumor-induced block in adipocyte differentiation is mediated by the selective inhibition of expression of the essential adipogenic transcription factors, namely, CCAAT/enhancer-binding protein alpha (C/EBPα) and peroxisome proliferator-activated receptor gamma (PPAR-γ) (Figure 1.12). The inhibition of differentiation of fibroblasts to mature adipocytes mediated by TNF and IL-11 is the key event responsible for desmoplastic reaction. Moreover, blocking both TNF and IL-11 in cancer cell conditioned medium using neutralizing antibodies is sufficient to reverse this antidifferentiative effect of cancer cells completely. (Meng et al, 2001).

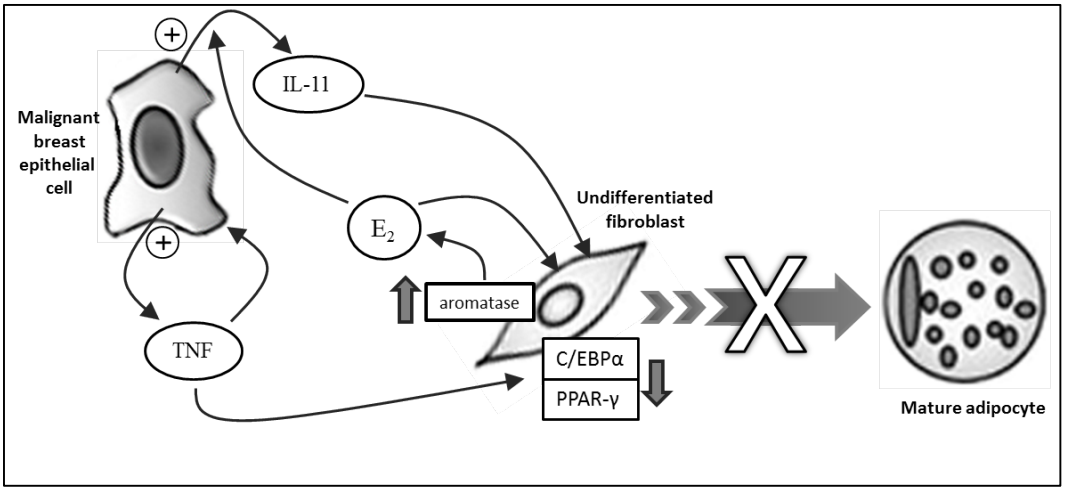

Figure 1.12: Detail of epithelial-stromal interaction via estrogen and cytokines in breast cancer.

The pathologic significance of local aromatase activity in breast cancer was also supported via in vitro studies. MCF7 breast cancer cells, which were stably transfected to express an mouse mammary tumor virus- promoter-driven human aromatase complementary DNA (cDNA) and inoculated into oophorectomized nude mice, remained dependent on circulating androstenedione for their rapid growth (Yue et al., 1994). Further evidence for the importance of local aromatase expression in the breast tissue came from an in vivo mouse model demonstrating that aromatase overexpression in breast tissue is sufficient for maintaining hyperplasia in the absence of circulating estrogen and that aromatase inhibitors abrogated hyperplasia (Tekmal et al., 1999). These transgenic mice with mouse mammary tumor virus promoter-driven local aromatase in breast tissue are more prone for breast cancer development (Kovacic et al., 2004).

1.5 The Scope of Current Study

In this study, local expressions of CYP17A1, CYP19A1 genes and activity levels of aromatase in invasive ductal breast carcinoma tissues were investigated by means of revealing their effect over peripheral and/or intratumoral estrogen production in invasive ductal breast carcinoma tissues. For this purpose, tumor and neighboring mammary adipose tissues from which is diagnosed pathologically as invasive ductal breast cancer were collected. CYP17A1, CYP19A1 expressions and aromatase activity were compared within the groups while healthy breast tissue group was used as control. Two different methods, a radiaimmunoassay (RIA) and a liquid

chromatography-tandem mass spectrometry (LC-MS/MS) based, were developed for the measurement of the aromatase activity. The results were evaluated further in view of the clinicopathological characteristics and breast cancer risk factors. When taken together, the present results revealed significant clues on possible mechanism of the estrogen dependent breast cancer initiation and progression in postmenapausal women, which is promising for estrogen driven risk estimations and patient specific treatment decision making in clinical practice.

2. MATERIAL AND METHODS

2.1 Materials

2.1.1 Patient selection and tissue samples

Tumor (T) and neighboring adipose tissues (P) were obtained from 20 female patients who did not receive adjuvant chemotherapy before surgery and underwent mastectomy or breast conserving surgery due to invasive ductal breast cancer at the department of Surgery, Cerrahpasa Faculty of Medicine in Istanbul University during the period of January 2010 – December 2012. Surgically resected tissues were subjected to pathological examination to diagnose and confirm the correct sampling of tumor and adipose tissue at department of Pathology, Cerrahpasa Faculty of Medicine in Istanbul University. One tumor and one neighboring mammary adipose tissue sample was obtained from each patient. They were snap frozen in liquid nitrogen and kept at -80°C until use for the determination of aromatase activity and for the extraction of total RNA. All patients were classified as luminal A (ER(+), PR (+), HER2 (-)) and postmenapausal. Only tumor cells with distinct nuclear immunostaining for ER and PR were recorded as positive. The ER and PR status of the patients were defined by immunohistochemistry on formalin-fixed, paraffin-embedded sections of clinical specimens as part of routine pathological interpretation. Immunohistochemistry was performed using a rabbit monoclonal antihuman ER antibody (clone SP1; Thermo-Scientific, MA, USA) and a polyclonal rabbit antihuman PR antibody (clone 16, Novocastra, Leica Microsystem, Wetzlar, Germany). Two different pathologists evaluated ER/PR immunohistochemical stainings. Nuclear staining of > 10% of cells were accepted as positive for ER or PR status. According to chromogen intensity, dark and intense staining of receptors was evaluated as strong intensity; otherwise, it was accepted as weak intensity. In addition 12 tumor-free breast tissue samples (N) were obtained from premenopausal women with no history of breast cancer (age range= 20-40 years) who underwent reduction mammoplasty surgery as the control group. None of them had any kind of cancer history (before surgery, breast ultrasonography was performed and after

surgery, specimens of these patients were all clean as pathologically) and any known metabolic disease. They attented to surgery outpatient clinic with thoracic, upper back and neck/shoulder pain or severe intertriginous dermatitis and cosmetic problems. Informed written consent about the study was obtained from each patient.

2.1.2 Chemicals

Chemicals used in this study are listed in Table 2.1.

Table 2.1: Chemicals.

Chemical	Producer
Dipotassium phosphate	Merck Millipore
Potassium dihydrogen phosphate	Merck Millipore
Sodium cholate	Applichem
Tween 20	Promega
Phenylmethylsulfonyl fluoride (PMSF)	Applichem
NADPH regenerating system (A-B)	BD Biosciences
Trichloroacetic acid	Sigma-Aldrich
Acetonitrile HPLC grade	Panreac
Trietilamine	Pierce
Ortophosphoric acid	Merck Millipore
Ethanol	Merck Millipore
Chloroform	Merck Millipore
Glycerol	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Applichem
DMSO	Calbiochem
Phosphate buffered saline (PBS)	Medicago
Estradiol	Sigma Aldrich
Testosterone	Sigma Aldrich
Estrone	Sigma Aldrich
17-β Estradiol Radioimmunoassay kit	MP Biomedicals
Methanol HPLC grade	Merck
Water HPLC grade	Merck

Table 2.1 (Continued): Chemicals.

Chemical	Producer
Trizol Reagent	Ambion
Purelink RNA Mini kit	Ambion
RNase away	Invitrogen
High Capacity RNA-to-cDNA Kit	Applied Biosystems
TaqMan® Gene Expression Assays	Applied Biosystems
Bicinchoninic acid (BCA) Protein assay	Pierce

2.1.3 Buffers and solutions

Buffers and solutions that were used in this study are listed in Table 2.2.

Table 2.2: Buffers and solutions.

Buffer/Solution	Concentration
PBS (pH: 7,4)	Working solution: 1X
Potassium phosphate buffer (pH: 7,4)	0,5 M
EDTA	2 mM
Sodium cholate	10 mM
PMSF	200 mM
Glycerol	20 % (v/v)
Tween 20	0,2 % (v/v)
Tricloroacetic acid	0,1 % (w/v)
Testosterone	5 mM

2.1.4 Laboratory equipments

Laboratory equipments that were used in this study are listed in Table 2.3.

Table 2.3: Laboratory equipments.

Laboratory Equipment	Producer
Autoclave	TOMY SX-700E High Pressure Steam Sterilizer
Deep freezers and refrigerators	-80℃ Sanyo Ultra Low Freezer -20℃ Arçelik +4℃ Arçelik
Spectrophotometer	NanoDrop ND-1000
Microplate spectrophotometer	Thermo Multiskan SPECTRUM
Agilent 1200 High performance liquid chromatography (HPLC) system	Agilent Technologies
HPLC Column	Agilent Technologies
Agilent 6460 Jet-Stream Triple Quad Mass Spectrometry (MS) System	Agilent Technologies
Solid Phase Extraction (SPE) Columns	Waters
Vacuum manifold	Agilent Technologies
Vacuum pump	Rocker Scientific Tanker 150
Incubator	Memmert UM 400
Microfuge	Nüve NF 048
Ultracentrifuge	Beckman Coulter ProteomeLab™ XL-A
Micropipettes	Thermo Scientific
pH meter	Mettler Toledo MP 220
Mortar and pestle	Haldenwanger
Homogenizer	Analytik Jena SpeedMill PLUS
Beads	Next Advance Zirconium 0,5mm
Real time PCR system	ABI 7500
Ultrapure water system	Millipore Milli-Q Plus PF
Ultracentrifuge	Beckman Coulter ProteomeLab™ XL-A
Micropipettes	Thermo Scientific

Table 2.3 (Continued): Laboratory equipments.

Laboratory Equipment	Producer
Water bath	Memmert WNB 10
Ultrasonic bath	Ultrasonic LC 30
Gamma counter	Packard Cobra 5000 Series B5003
Heating block	Biosan CH-100
Vortex	LMS VTX-3000L
Analytical balance	Libror AEX-200G
Heated magnetic stirrer	Heidolph MR 3001

2.2 Methods

Overview of current study's workflow can be summarized as in Figure 2.1.

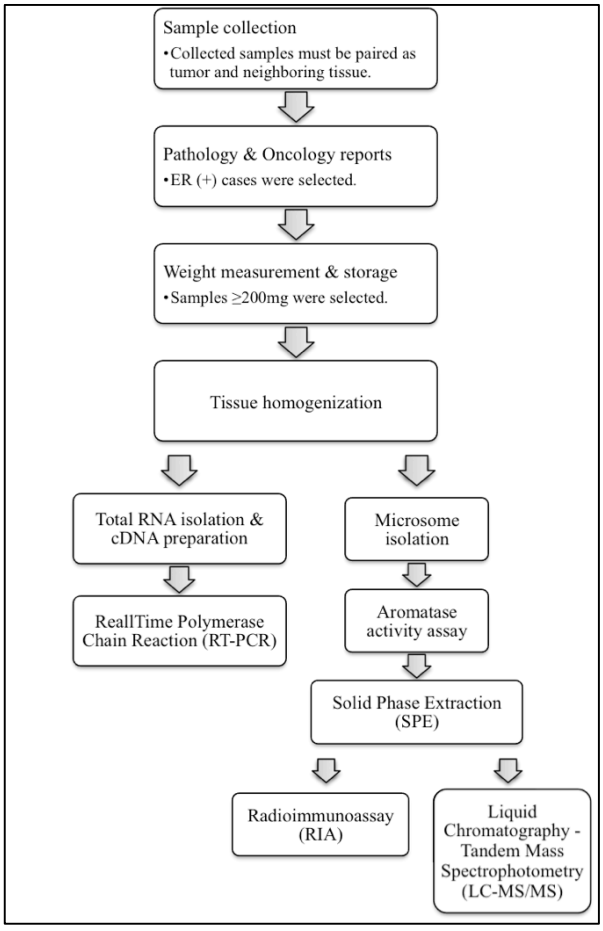

Figure 2.1: Workflow of the methods which were used in present study.

2.2.1 Total RNA isolation from tumor and neighbouring adipose tissues

Approximately 100-150 mg of tissue was grinded with liquid nitrogen using a ceramic mortar and pestle. TRIzol® reagent (Ambion) was used in conjunction with the PureLink® RNA Mini Kit (Ambion) to isolate total RNA from samples. A modification was made in manufacturer's protocol. We added an extra centrifugation step before choloroform addition in order to avoid excess fat content of the samples, which effects the RNA yields and interferes qRT-PCR. (Figure 2.2.). Determination of RNA concentrations following RNA isolation was done by using NanoDrop 1000 Spectrophotometer (Thermo Scientific).

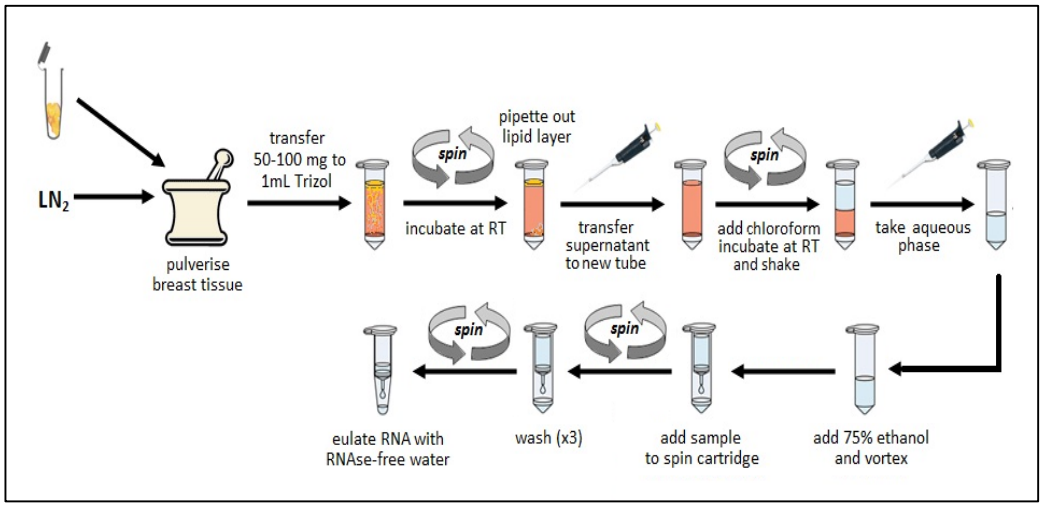

Figure 2.2: Modified RNA isolation method.

2.2.2 Analysis of expression levels of CYP17A1 and CYP19A1 by qRT-PCR

cDNA synthesis from isolated total RNA was performed according to High Capacity RNA-to-cDNA Kit protocol (Applied Biosystems). The sequences of primers and probes were shown in Table 2.4. Total RNA concentration was adjusted to 500 ng/reaction. qRT-PCR analysis was performed according to protocol of TaqMan® Gene Expression Assay (Applied Biosystems) kit by using Applied Biosystems® 7500 Real-Time PCR. Primer and probe sequences used in qRT-PCR analysis were shown in Table 2.4.

Carboxyfluorescein (FAM) reporter dye and minor groove binder (MGB) were attached at the 5' and 3' end of each probe, respectively. Briefly, 20 µl total PCR reaction contained; 1 µl of 20× TaqMan® Gene Expression Assay, cDNA, 10 µl of 2× TaqMan® Gene Expression Master Mix, 4 µl of cDNA template and 2 µl

RNase-free water. PCR reaction was conducted as follows: 50°C (2 min) for Uracil N-glycosylase (UNG) incubation, 95°C (10 min) for polymerase activation and then 40 cycles at 95°C (15 sec) / 60°C (1 min). TATA binding box protein (TBP) gene was used as the internal control group. The gene expression levels analysed ΔΔCt method (Livak and Schmittgen, 2001).

Table 2.4: Primer and probe sequences used in qRT-PCR analysis

	CYP17A1	CYP19A1
Forward	5'-TCACAATGAGA AGGAGTGGCAC -3'	5′-TGTGGACGTG TTGACCCTTCT-3′
Reverse	5'-TACTGACGGTG AGATGAGCTGG -3'	5′-ACCACGATAG CACTTTCGTCCA-3′
Probe	5'- TGCCTGAGCGTT TCTTGAATCCAGC-3'	5′-ATGCTGGACACCTCT AACACGCTCTTCTTGA-3′

2.2.3 Isolation of microsomes by differential centrifugation

Weighed tissues were pulverized with liquid nitrogen using a ceramic mortar and pestle and transferred to 1,5 mL microcentrifuge tubes containing 0.5mm diameter zirconium beads (Next Advance) and homogenization buffer (1x PBS (pH 7.4), 20% gliserol, 1 mmol/L EDTA). 1 mL of homogenization buffer was used for each 100mg of tissue sample. PMSF (0,2 mmol/L) was added just before homogenization. Homogenization was performed with SpeedMill Plus (Analytik Jena). The microsomal fraction was prepared with 105,000 g pellet which obtained from 9,000 g supernatant of the homogenate. The pellet obtained was resuspended in solubilization buffer (1x PBS (pH 7.4), 20% gliserol, 1 mmol/L EDTA, 1mmol/L sodium cholate and 0,2% Tween 20) and allowed to stand at 4°C overnight.

2.2.4 Bicinchoninic acid (BCA) protein assay

The Thermo Scientific Pierce BCA Protein Assay was used for the colorimetric detection and quantitation of total protein in patient samples. Assay was conducted according to manufacturer's instructions. Bovine serum albumine (BSA) was used as standart. 25µL of each standard or patient sample replicate was pipetted into a microplate well (working range = 20-2000µg/mL). 200µL of the working reagent was added to each well and plate was mixed thoroughly on a plate shaker for 30 seconds. Plate was covered and incubated at 37°C for 30 minutes and cooled to room temperature. Absorbance was measured at 562nm on the plate reader (Thermo

Multiskan SPECTRUM). the average 562nm absorbance measurement of the blank standard replicates were subtracted from the 562nm measurements of all other individual standard and patient sample replicates. A standard curve was prepared by plotting the average blank-corrected 562nm measurement for each BSA standard vs. its concentration in μg/mL. The standard curve was used to determine the protein concentration of each sample.

2.2.5 Aromatase activity assay

Aromatase activity assay was performed as described previously (Zharikova et al., 2006). Briefly, the activity of microsomal fractions in catalyzing the conversion of testosterone to 17-ß estradiol (E_2) was determined in a total reaction volume of 1 mL potassium phosphate buffer (0,5M pH 7.4). Testosterone (total reaction concentration 0,5 μg/mL) were added to the reaction solution and preincubated for 5 min at 37C. The reaction was initiated by the addition of NADPH regenerating system (NADP 0.4 mM, glucose-6-phosphate (G-6-P) 4 mM, G-6-P dehydrogenase 1 U/mL, and 2 mM $MgCl_2$) and incubated for 5 min at the same temperature. The reaction was terminated by the addition of 100 μL of a 10% (w/v) trichloroacetic acid and placed on ice. Estrone (E_1), 0,5 μL of 100μg/mL solution, was spiked to each tube as an internal standard. In addition a blank sample was prepared with same materials and conditions but without substrate.

2.2.6 Solid phase extraction (SPE) of the samples

The solid phase extraction protocol (Newman et al., 2008) was used with slight modifications, for extraction of 17-ß estradiol formed after aromatase activity assay. Briefly, protocol involves six major steps: (1) Solvation, columns were primed with ethanol; (2) Equilibration, water was passed through columns to prepare sorbent for sample loading; (3) Sample loading, samples in aqueous matrix were passed through the columns; (4) Interference elution, water was passed through columns to wash out interfering polar substances; (5) Sample elution, estradiol was eluted from columns with a small amount of eluant into 7 mL glass tubes; (6) Drying & storing, eluates were dried and stored at – 20°C (Figure 2.3).

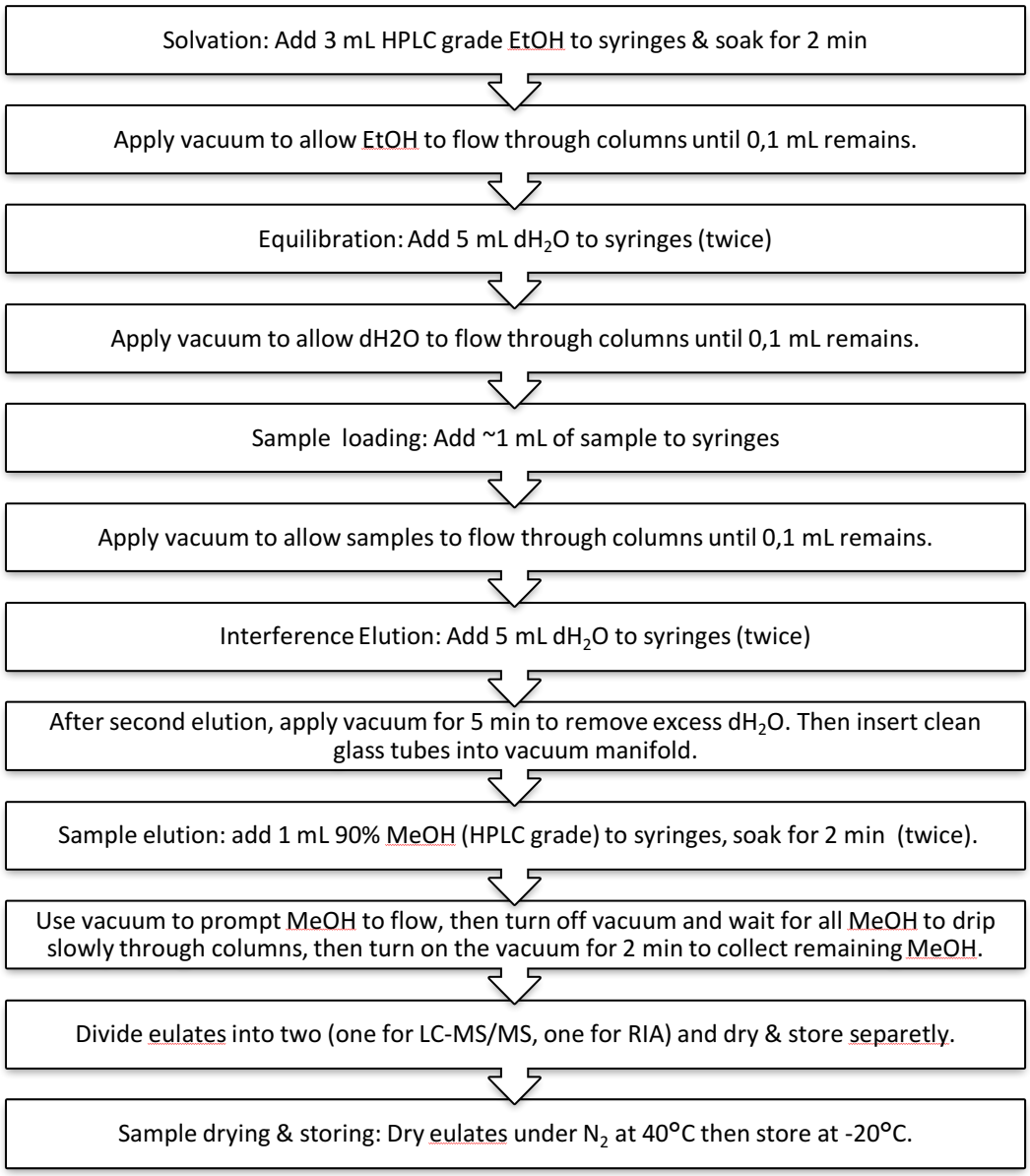

Figure 2.3: SPE procedure applied to the samples.

For SPE, a 20-place vacuum manifold (Vac Elut 20, Agilent Technologies), endcapped C18 columns (Sep-Pak C18 Plus Short Cartridge, 360 mg sorbent, 55-105 µm particle size, Waters), and 10 mL disposable syringes without needles were used (Figure 2.4).

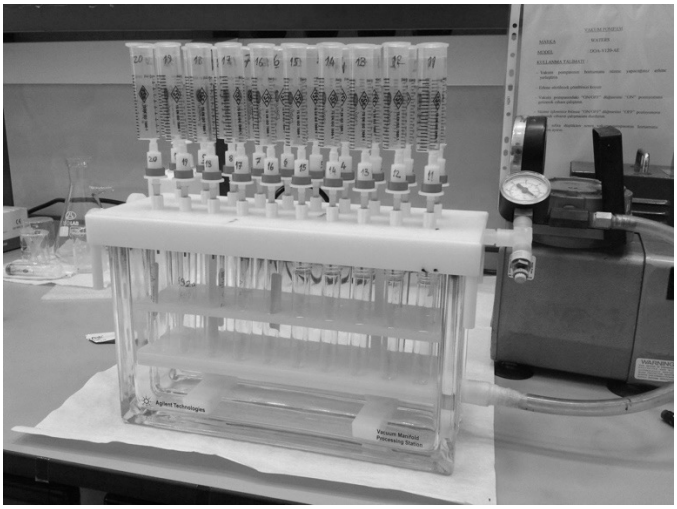

Figure 2.4: SPE set up for the extraction of samples after activity experiment.

2.2.7 Measurement of E_2 formation

E_2 formation was measured for the calculation of specific aromatase activity in the samples. Measurements were performed with two different methods: RIA and LC-MS/MS.

2.2.7.1 Measurement via RIA

For detection of E_2 formed in samples, ImmuChem Double Antibody ^{125}I RIA kit for 17ß-Estradiol (MP Biomedicals) was used. This kit was desinged for in vitro diagnostic use in serum or plasma samples. Kit's protocol was followed but only sample preperation procedure of the kit was modified for aromatase activity assay samples. Evaporated extracts of aromatase assay samples were resuspended and dilluted 100 fold with standart 1 (0 pg/mL). Then 50 µL of it used for each related tube. 100, 1000 and 2500 pg/mL E_2 were prepared with standart 1 and used as controls. All standarts, controls and unknown samples were studied double. E_2 measurements were performed with a gamma counter (Packard Cobra 5000 Series-B5003) at Istanbul University School of Medicine, Endocrinology Department.

2.2.7.2 Measurement via LC-MS/MS

Standart and sample preparation

Working standards of E_1, E_2 and TES were prepared by diluting 1 mg/mL stock solutions in methanol. Calibrators containing E_1, E_2 and TES in concentrations of 1,

10, 100, 250, 500 and 1000 ppb were prepared in the same matrix. Perivously evaporated and stored samples dissolved in 100 µL methanol before injection.

LC-MS/MS conditions

LC–MS/MS analysis was carried out on a Agilent 6460 Jet-Stream Triple Quad System with Agilent 1200 Series HPLC system. A C18 reversed-phase column (3.0×30mm; 1.8µm, Zorbax SB-C18) with an online filter was used for chromatographic separation. HPLC conditions are shown in Table 2.5 and MS/MS instrument parameters are shown in Table 2.6. The data were collected and quantified using Agilent Mass Hunter software.

Table 2.5: HPLC conditions.

Time (min)	**Solvent A (%) (Mobile Phase A)**	**Solvent B (%) (Mobile Phase B)**	**Flow (ml/min)**
0	100	0	0.6
2.60	90	10	0.6
2.70	60	40	0.6
4.00	60	40	0.6
Solvent A		ACN + 0.1% (v/v) formic acid	
Solvent B		H_2O + 0.1% (v/v) formic acid	
Column temprature		40 °C	
Autosampler		Set a 2 sec needle wash, using the flushport	
Injection volume		1µL	

Table 2.6: MS/MS conditions.

Ion source	ESI (Agilent Jet Stream)
Polarity	Positive
Gas Temperature	350 °C
Gas Flow	13 L/min
Nebulizer Pressure	40 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	6000 V (positive)
Nozzle Voltage	2000 V (positive)
Resolution MS1 and MS2	Wide resolution

2.2.8 Statistical analysis

We conducted our analysis among three groups (P: Peripheral, T: Tumor, N: Healthy), which were grouped according to the tissue type. We especially focused our analysis on peripheral tissue group due to the hypothesis that it is the source of aromatase activity in breast cancer tissue microenvironment. All calculations were performed using SPSS Statistical Program Version 21.0 (SPSS Inc. Chicago, USA). The significance of differences in mRNA levels and aromatase activities were determined by the Wilcoxon Signed Ranks (WSR) test, Manwhitney U (MU) test or McNemar (MN) test as needed. Moreover, kappa values (κ) were obtained and results were interpreted according to the study previously reported (Landis and Koch 1977). Briefly κ ranges generally from 0 to 1.0 (although negative numbers are possible) where large numbers meaning better reliability, values near or less than 0 suggest that the agreement is attributable to chance alone. All reported p-values are from two-sided tests and a value less than 0.05 was considered statistically significant.

3. RESULTS

3.1 Clinical Characteristics of Selected Patients

The mean age (± SD) of the study group (n=20) was 59.65± 11.94 years. Most patients had disease of histological grade II or higher (17/20, 85.0%) and stage of the disease is IIa or worse (15/20, 75.0%) The primary characteristics of the study population are given in Table 3.1.

Table 3.1: Clinicopathological characteristics of patients (n=20).

Patient characteristics	Mean±SD	Range
Age (years)	59.65 ±11.94	46 - 87
BMI (kg/m^2)	27.871 ±5.06	22.3 - 37.0
	Status	n (%)
Histological grade	I	3 (15.0%)
	II	12 (60.0%)
	III	5 (25.0%)
Stage	Ia	5 (25.0%)
	IIa	7 (35.0%)
	IIIa	2 (10.0%)
	IIIc	4 (20.0%)
	IV	2 (10.0%)
Lymphatic vascular invasion	Yes	10 (50.0%)
	No	10 (50.0%)
Axilla invasion	Yes	7 (35.0%)
	No	13 (65.0%)
Insitu component	Yes	18 (90.0%)
	No	2 (10.0%)
Blood vessel invasion	Yes	0 (0.0%)
	No	20 (100.0%)
Necrosis	Yes	6 (30.0%)
	No	14 (70.0%)
Perineural invasion	Yes	13 (65.0%)
	No	7 (35.0%)
Calcification	Yes	7 (35.0%)
	No	13 (65.0%)
Ki67	Yes	14 (70.0%)
	No	6 (30.0%)

3.2 Breast Cancer Risk Factors

The study group was homogeneous according to receptor status and disease type which all patients were ER (+) PR (+) and diagnosed as invasive ductal carcinoma. Half of the group have lymphatic vascular invasion. None of the patients had blood

vessel invasion and more than half (65.0%, 70.0% and 65.0%) were lack of axilla invasion, necrosis and calcification, respectively. However, presence of an in situ component, perineural invasion and Ki67 were detected in most of the cases. According to indicated risk factors, more than half of the patients bearing the "low risk" conditions, except body mass index (BMI), oral contraceptive use and age at menopause (Table 3.2).

Table 3.2: Breast cancer risk factor distribution among the study group.

Risk factors	Patients (n = 20)	
	High risk	**Low risk**
Body mass index (BMI-kg/m2)	≥ 25	< 25
	11 (55%)	9 (45%)
Family history of cancer	Yes	No
	5 (25%)	13 (65%)
History of cancer in breast	Yes	No
	4 (20%)	16 (80%)
First-degree relatives with breast cancer	Yes	No
	3 (15%)	15 (75%)
Age at first full-term pregnancy	≥ 30	< 30
	4 (20%)	12 (60%)
Smoking	Yes	No
	8 (40%)	12 (60%)
Alcohol intake	Yes	No
	6 (30%)	14 (70%)
Age at menarche	< 12	≥ 12
	4 (20%)	16 (80%)
Parity	Nulliparous	Multiparous
	4 (20%)	16 (80%)
Breast feeding	No	Yes
	-	16 (80%)
Age at menopause	≥ 45	< 45
	17 (85%)	3 (15%)
Oral contraceptive use	Yes	No
	2 (10%)	18 (90%)

3.3 CYP17A1 and CYP19A1 Expressions

qRT-PCR analysis was performed according to protocol of TaqMan® Gene Expression Assay (Applied Biosystems) kit by using Applied Biosystems® 7500 Real-Time PCR. The gene expression levels calculated according to the $2^{-(\Delta\Delta Ct)}$ method are shown in Table 3.3.

Table 3.3: The expression levels of CYP17A1 and CYP19A1 genes normalized with TATA Binding Box Protein (TBP) housekeeping gene, as determined by qRT- PCR.

Sample name	CYP17A1-TBP ΔCt Mean	ΔΔCt	$2^{-(\Delta\Delta Ct)}$	CYP19A1-TBP ΔCt Mean	ΔΔCt	$2^{-(\Delta\Delta Ct)}$
N	10.60	0.00	1.00	14.87	0.00	1.00
P01	10.63	0.02	0.98	11.99	-2.89	7.39
P02	11.20	0.60	0.66	17.28	2.41	0.19
P03	10.22	-0.38	1.70	16.13	1.26	0.42
P04	6.97	-3.64	12.43	11.52	-3.35	10.23
P05	9.99	-0.62	1.53	13.99	-0.88	1.84
P06	8.88	-1.72	3.30	11.62	-3.25	9.54
P07	13.38	2.77	0.15	11.58	-3.30	9.82
P08	10.55	-0.05	1.04	14.49	-0.38	1.30
P09	9.10	-1.50	2.84	10.93	-3.94	15.37
P10	10.21	-0.39	1.31	14.86	-0.01	1.01
P11	9.26	-1.34	2.54	13.93	-0.94	1.92
P12	11.40	0.80	0.58	15.74	0.87	0.55
P13	14.00	3.40	0.09	14.21	-0.66	1.58
P14	5.82	-4.78	27.55	10.02	-4.85	28.89
P15	12.28	1.68	0.31	10.74	-4.13	17.54
P16	13.04	2.44	0.185	12.13	-2.74	6.69
P17	12.87	2.27	0.21	18.65	3.78	0.07
P18	15.06	4.46	0.05	13.68	-1.19	2.29
P19	12.61	2.01	0.25	13.47	-1.40	2.64
P20	11.56	0.96	0.52	15.29	0.42	0.75
T01	10.30	-0.30	1.23	17.04	2.17	0.22
T02	15.07	4.47	0.05	18.49	3.62	0.08
T03	8.97	-1.63	3.10	19.79	4.92	0.03
T04	7.55	-3.05	8.31	13.03	-1.84	3.59
T05	7.26	-3.34	10.15	16.21	1.34	0.40
T06	10.49	-0.11	1.08	15.70	0.83	0.56

Table 3.3 (Continued): The expression levels of CYP17A1 and CYP19A1 genes normalized with TATA Binding Box Protein (TBP) housekeeping gene, as determined by qRT- PCR.

Sample name	CYP17A1-TBP ΔCt Mean	ΔΔCt	$2^{-(\Delta\Delta Ct)}$	CYP19A1-TBP ΔCt Mean	ΔΔCt	$2^{-(\Delta\Delta Ct)}$
T07	11.72	1.12	0.46	12.21	-2.66	6.33
T08	13.59	2.99	0.13	14.79	-0.08	1.06
T09	7.75	-2.85	7.23	11.44	-3.43	10.80
T10	18.50	7.90	0.004	16.20	1.33	0.40
T11	14.46	3.86	0.07	15.07	0.20	0.87
T12	15.17	4.57	0.04	20.24	5.37	0.02
T13	11.78	1.18	0.44	13.04	-1.83	3.56
T14	5.98	-4.62	24.66	11.46	-3.41	10.65
T15	7.06	-3.54	11.66	13.18	-1.69	3.23
T16	13.11	2.51	0.176	14.74	-0.13	1.10
T17	12.97	2.37	0.19	20.14	5.27	0.03
T18	12.02	1.42	0.37	18.53	3.66	0.21
T19	11.77	1.17	0.45	16.14	1.27	0.86
T20	12.70	2.10	0.23	16.18	1.31	0.98

According to tissue types, CYP17A1 expression levels were ordered as N>P>T. CYP19A1 was highly expressed in P compared to both other types. Among all cases, there was a strong upregulation of CYP19A1 and a slight upregulation of CYP17A1 in peripheral tissue compared to paired tumor tissues (Figure 3.1).

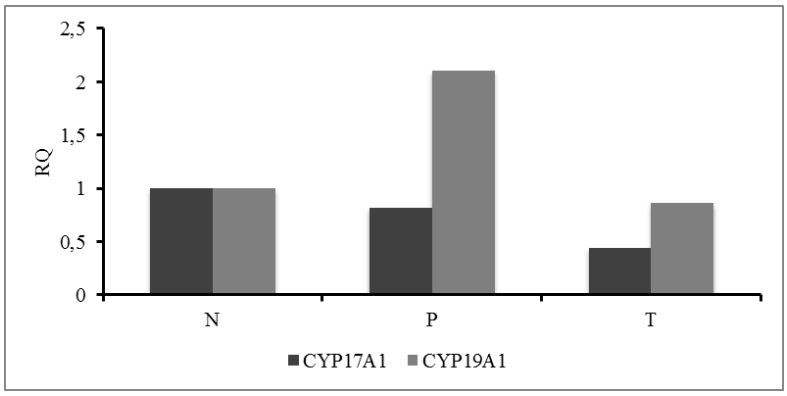

Figure 3.1: Overall expressions of CYP17A1 and CYP19A1 in tissue groups

Nearly 3 fold higher CYP19A1 expression levels were observed in peripheral tissues compared to tumor tissues (Z= -3.397, p=0.001 [WSR]). However the upregulation of CYP17A1 was unable to reach statistical significance (Z= -0.402, p=0.687 [WSR]) (Figure 3.2).

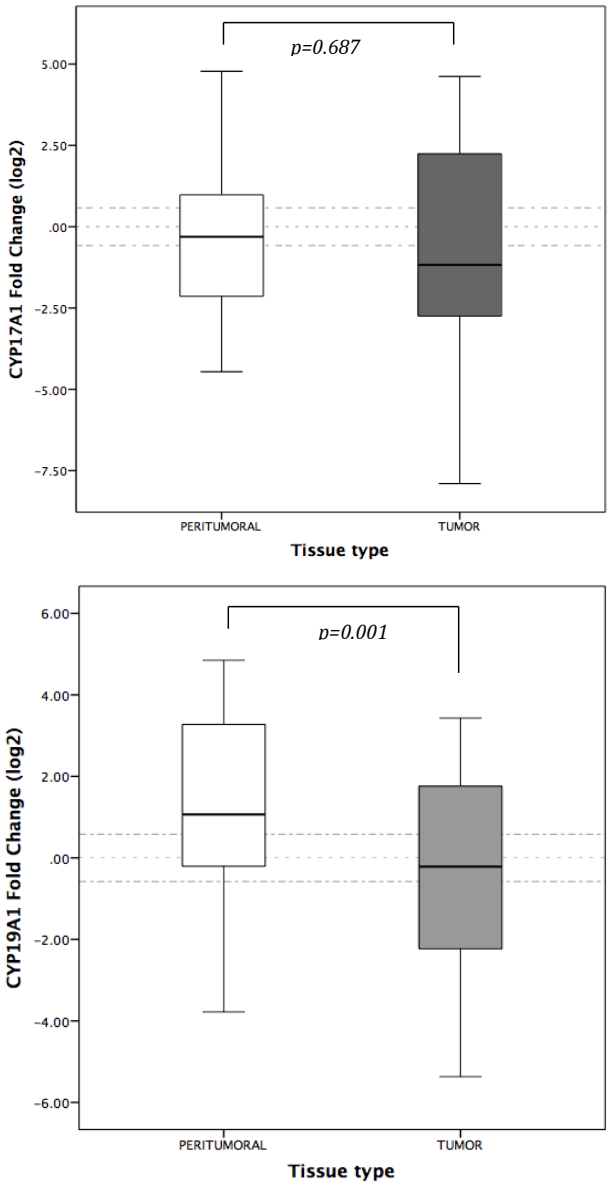

Figure 3.2: Box plots of relative qRT-PCR gene expression measurements of CYP17A1 and CYP19A1 in peripheral and tumor breast tissues. The dotted lines represent the cut-off value which is 1,5 for gene expression fold changes.

The relative expression levels of CYP17A1 and CYP19A1 of individual patients are shown in Figure 3.3. Healthy tissue sample expression levels were used as controls.

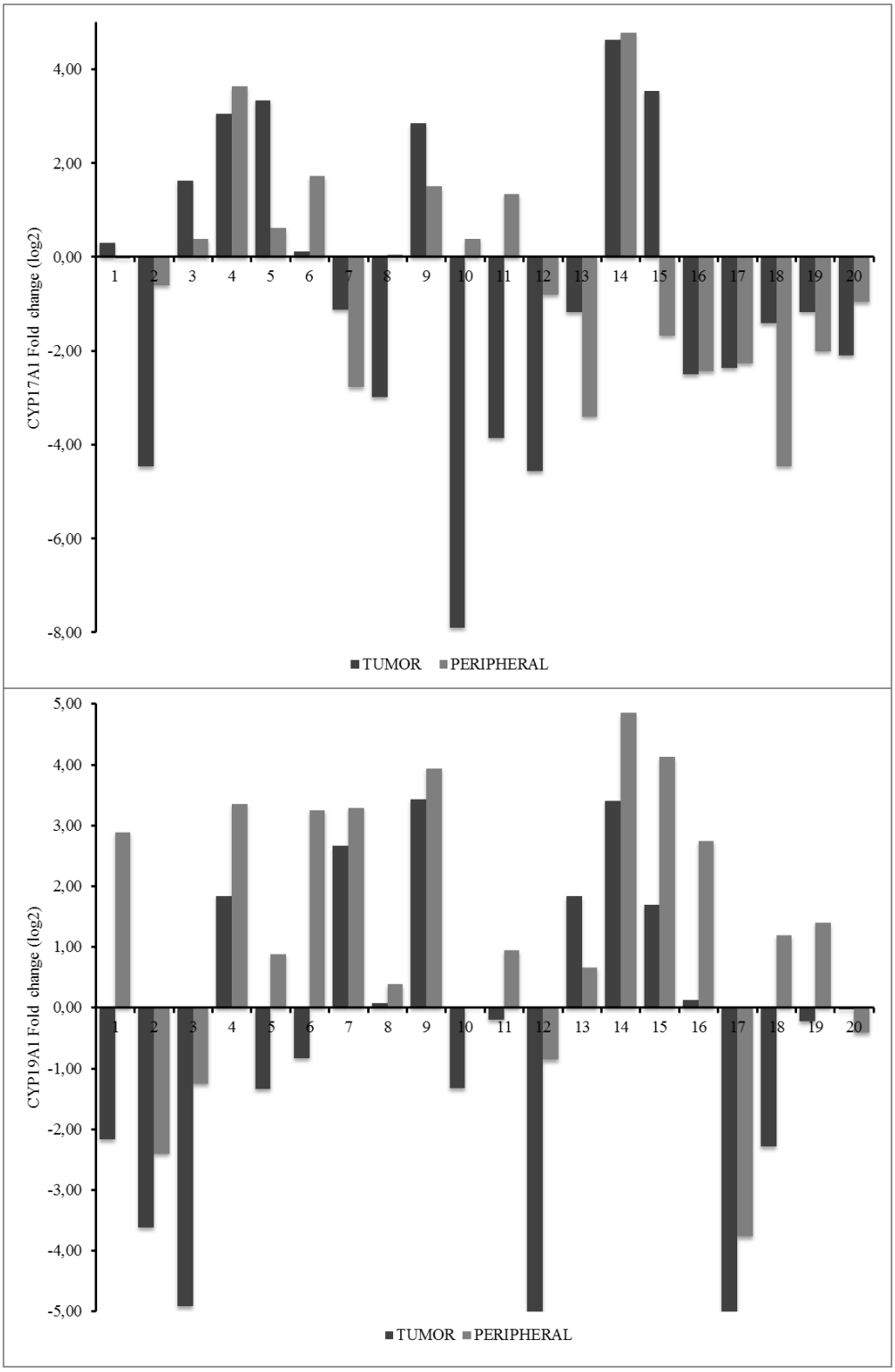

Figure 3.3: The fold change of CYP17A1 and CYP19A1 gene in peripheral and tumor tissue of each patient. Results shown as fold change (log 2 relative quantification (RQ)). The healthy tissue group was used as reference.

Table 3.4 and Table 3.5 summarize the significant associations of patient characteristics with CYP17A1 and CYP19A1 expression levels in peripheral tissue compared to healthy and tumor tissue, respectively.

Table 3.4: Tissue CYP17A1 expression levels for the patient characteristics. P vs. N: Peripheral tissue compared to healthy breast tissue, P vs. T: Peripheral tissue compared to tumor tissue, PR: Progesterone receptor. (*) All patients have positive PR staining status, with different intensity. Nuclear staining of > 10% of cells were accepted as positive for ER or PR status.

Patient characteristic		P vs N		P vs T	
		Up regulated n (%)	*Down regulated / No alteration* n (%)	Up regulated n (%)	*Down regulated / No alteration* n (%)
Histological grade	I	1 (33.3)	2 (66.7)	1 (33.3)	2 (66.7)
	II+III	5 (29.4)	12 (70.6)	7 (41.2)	10 (58.2)
	p-Value (kappa)	0.003 (-0.016)		0.012 (0.035)	
Stage	I	1 (20.0)	4 (80.0)	1 (20.0)	4 (80.0)
	Others	5 (33.3)	10 (66.7)	7 (46.7)	8 (53.3)
	p-Value (kappa)	0.012 (0.083)		0.039 (0.182)	
In situ component	Yes	6 (33.3)	12 (66.7)	8 (44.4)	10 (55.6)
	No	-	2 (100.0)	-	2 (100.0)
	p-Value (kappa)	0.0005 (0.091)		0.002 (0.138)	
PR status*	Weak intensity	-	5 (100.0)	1 (20.0)	4 (80.0)
	Strong intensity	6 (40.0)	9 (60.0)	7 (46.7)	8 (53.3)
	p-Value (kappa)	0.004 (0.250)		0.039 (0.182)	
Age	≥ 50	4 (28.6)	10 (71.4)	5 (35.7)	9 (64.3)
	< 50	2 (33.3)	4 (66.7)	3 (50.0)	3 (50.0)
	p-Value (kappa)	0.039 (-0.034)		0.146 (-0.111)	
Parity	*Nulliparous*	2 (50.0)	2 (50.0)	1 (25.0)	3 (75.0)
	Multiparous	4 (25.0)	12 (75.0)	7 (43.8)	9 (56.2)
	p-Value (kappa)	0.013 (-0.129)		0.021 (0.107)	
Age at menopause	*≥ 45*	5 (29.4)	12 (70.6)	8 (47.1)	9 (52.9)
	< 45	1 (33.3)	2 (66.7)	-	3 (100.0)
	p-Value (kappa)	0.003 (-0.016)		0.004 (0.212)	

Table 3.5: Tissue CYP19A1 expression levels for the patient characteristics. P vs. N: Peripheral tissue compared to healthy breast tissue, P vs. T: Peripheral tissue compared to tumor tissue.

Patient characteristic		P vs N		P vs T	
		Up regulated n (%)	Down regulated / No alteration n (%)	Up regulated n (%)	Down regulated / No alteration n (%)
Axilla invasion	Yes	5 (71.4)	2 (28.6)	7 (100.0)	-
	No	8 (61.5)	5 (38.5)	7 (53.8)	6 (46.2)
	p-Value (kappa)	0.109 (0.083)		0.016 (0.375)	
Family history of cancer	Yes	4 (80.0)	1 (20.0)	4 (80.0)	1 (20.0)
	No	8 (61.5)	5 (38.5)	8 (61.5)	5 (38.5)
	p-Value (kappa)	0.039 (0.129)		0.039 (0.129)	
History of cancer	Yes	-	4 (100.0)	2 (50.0)	2 (50.0)
	No	13 (81.3)	3 (18.7)	12 (75.0)	4 (25.0)
	p-Value (kappa)	0.049 (-0.441)		0.013 (-0.129)	
Age at first full-term pregnancy	≥ 30	3 (75.0)	1 (25.0)	3 (75.0)	1 (25.0)
	< 30	8 (66.7)	4 (33.3)	7 (58.3)	5 (41.7)
	p-Value (kappa)	0.039 (0.053)		0.070 (0.111)	
Age at menarche	*< 12*	-	4 (100.0)	1 (25.0)	3 (75.0)
	≥ 12	13 (81.3)	3 (18.7)	13 (81.3)	3 (18.7)
	p-Value (kappa)	0.049 (-0.441)		0.021 (-0.290)	
Oral contraceptive use	*Yes*	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)
	No	12 (66.7)	6 (33.3)	13 (72.2)	5 (27.8)
	p-Value (kappa)	0.003 (-0.048)		0.002 (-0.061)	

We created two groups according to the common findings in literature (O'Neill et al., 1988; Bulun et al., 1993; Harada et al., 1993; Reed et al., 1993; Sasano et al., 1994; Utsumi et al., 1996; Zhou et al., 1996; Miyoshi et al., 2003) of how these CYPs' mRNAs act over local estrogen production in breast, estrogen increasing (IP) and estrogen decreasing (DP) pattern, by combining both CYPs' expression status (Table 3.6). The significant associations of DP and IP conditions with patient characteristics are analayzed and shown in Table 3.7.

Table 3.6: Distribution of CYP17A1 and CYP19A1 expression status together by threshold value (≥1.5-fold change) for different tissue comparison groups. P vs. N: Peripheral tissue compared to healthy breast tissue, P vs. T: Peripheral tissue compared to tumor tissue.

Group	mRNA alteration (CYP17A1/CYP19A1)	P vs. N (n= 20)	P vs. T (n= 20)
Local Estrogen Increasing Expressional Pattern (IP)	up/up	6 (30.0%)	5 (25.0%)
	up/unaltered	1 (5.0%)	4 (20.0%)
	Unaltered/up	-	2 (10.0%)
Local Estrogen Decreasing Expressional Pattern (DP)	up/down	6 (30.0%)	5 (25.0%)
	Unaltered/down	1 (5.0%)	1 (5.0%)
	Down/down	3 (15.0%)	2 (10.0%)
	Down/up	-	1 (5.0%)
	Down/unaltered	1 (5.0%)	-
	Unaltered/unaltered	2 (10.0%)	-

Table 3.7: The combined effect of CYP17A1 and CYP19A1 expression levels for different subgroups of patient characteristics. DP: Local estrogen production decreasing expressional pattern, IP: Local estrogen production increasing expressional pattern, P vs. N: Peripheral tissue compared to healthy breast tissue, P vs. T: Peripheral tissue compared to tumor tissue, PR: Progesterone receptor. (*) All patients have positive PR staining status, with different intensity. Nuclear staining of > 10% of cells were accepted as positive for ER or PR status.

Patient characteristic		P vs N		P vs T	
		DP n (%)	IP n (%)	DP n (%)	IP n (%)
Histological grade	I	1 (33.3)	2 (66.7)	1 (33.3)	2 (66.7)
	II+III	6 (35.3)	11 (64.7)	10 (58.8)	7 (41.2)
	p-Value (kappa)	0.006 (0.008)		0.070 (0.140)	
Stage	I	1 (20.0)	4 (80.0)	1 (20.0)	4 (80.0)
	Others	6 (40.0)	9 (60.0)	10 (66.7)	5 (33.3)
	p-Value (kappa)	0.021 (0.130)		0.216 (0.368)	
In situ component	Yes	7 (38.9)	11 (66.1)	11 (66.1)	7 (38.9)
	No	-	2 (100.0)	-	2 (100.0)
	p-Value (kappa)	0.001 (0.113)		0.016 (0.239)	
PR status*	Weak intensity	1 (20.0)	4 (80.0)	3 (60.0)	2 (40.0)
	Strong intensity	6 (40.0)	9 (60.0)	8 (53.3)	7 (46.6)
	p-Value (kappa)	0.021 (0.130)		0.344 (-0.053)	
Parity	*Nulliparous*	2 (50.0)	2 (50.0)	8 (50.0)	8 (50.0)
	Multiparous	5 (31.2)	11 (68.8)	3 (75.0)	1 (25.0)
	p-Value (kappa)	0.022 (-0.102)		0.227 (-0.170)	
Age at menopause	*≥ 45*	6 (35.3)	11 (64.7)	1 (33.3)	2 (66.7)
	< 45	1 (33.3)	2 (66.7)	10 (58.8)	7 (41.2)
	p-Value (kappa)	0.006 (0.008)		0.070 (0.140)	
Oral contraceptive use	*No*	-	2 (100.0)	10 (55.6)	8 (44.4)
	Yes	7 (38.9)	11 (66.1)	1 (50.0)	1 (50.0)
	p-Value (kappa)	0.180 (-0.180)		0.012 (-0.019)	

There was no statistically significant association between factors such as BMI, smoking, alcohol consumption, lymphovascular invasion, perineural invasion, necrosis, calcification, Ki67 and expressional differences neither as individual nor combined (DP and IP) in P, T or N group.

3.4 Protein Concentrations

Protein measurement analysis was performed according to the Pierce BCA Protein Assay (Thermo Scientific) protocol by using the Multiskan Spectrum plate reader (Thermo Scientific). Figure 3.4 shows the calibration graph, while Table 3.8 shows sample protein concentrations calculated via this graph.

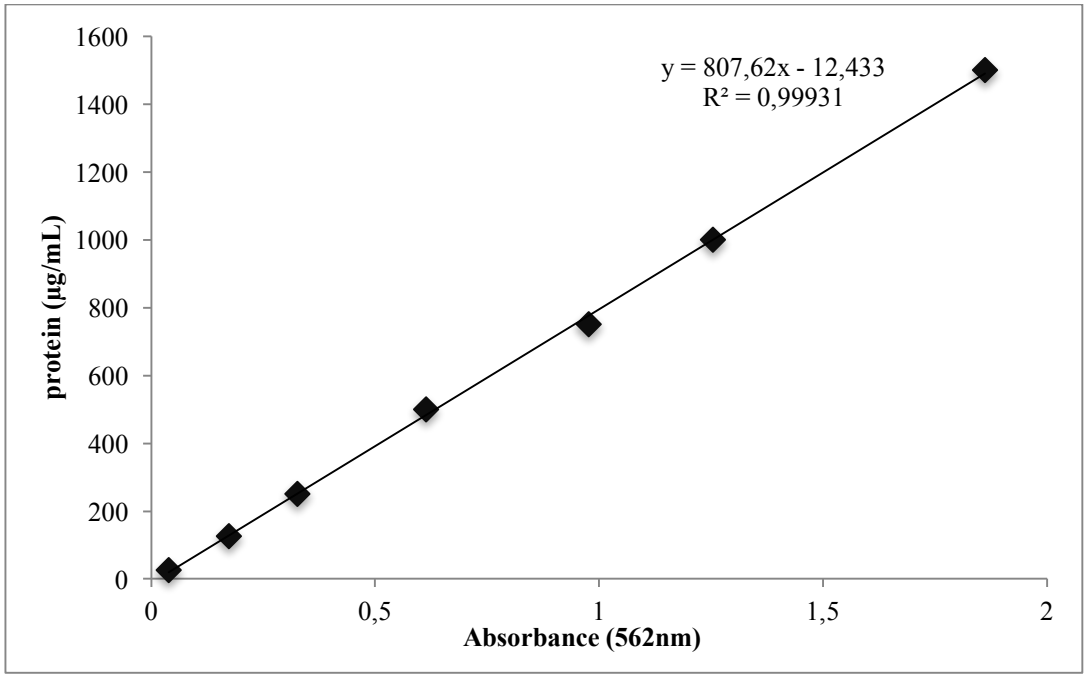

Figure 3.4: BCA protein assay calibration graph.

Table 3.8: Protein concentrations of the samples.

Sample name	Protein (µg/mL)	Sample name	Protein (µg/mL)
N	112,99	T01	250,55
P01	39,73	T02	231,16
P02	16,99	T03	136,44
P03	86,64	T04	137,11
P04	109,95	T05	75,46
P05	2,74	T06	156,64
P06	59,84	T07	247,16
P07	42,30	T08	377,87
P08	6,87	T09	257,86
P09	183,00	T10	425,48
P10	215,74	T11	337,76
P11	94,03	T12	215,44
P12	9,00	T13	170,06
P13	78,80	T14	181,05
P14	28,27	T15	58,88
P15	126,82	T16	311,45
P16	168,29	T17	989,12
P17	156,09	T18	809,74
P18	39,09	T19	401,68
P19	186,78	T20	333,84
P20	442,65		

3.5 Aromatase Activity

3.5.1. RIA measurements

RIA measurements were conducted as previously described in Methods section. The calibration graph for standarts between 10-3000 pg/mL is shown in Figure 3.5.

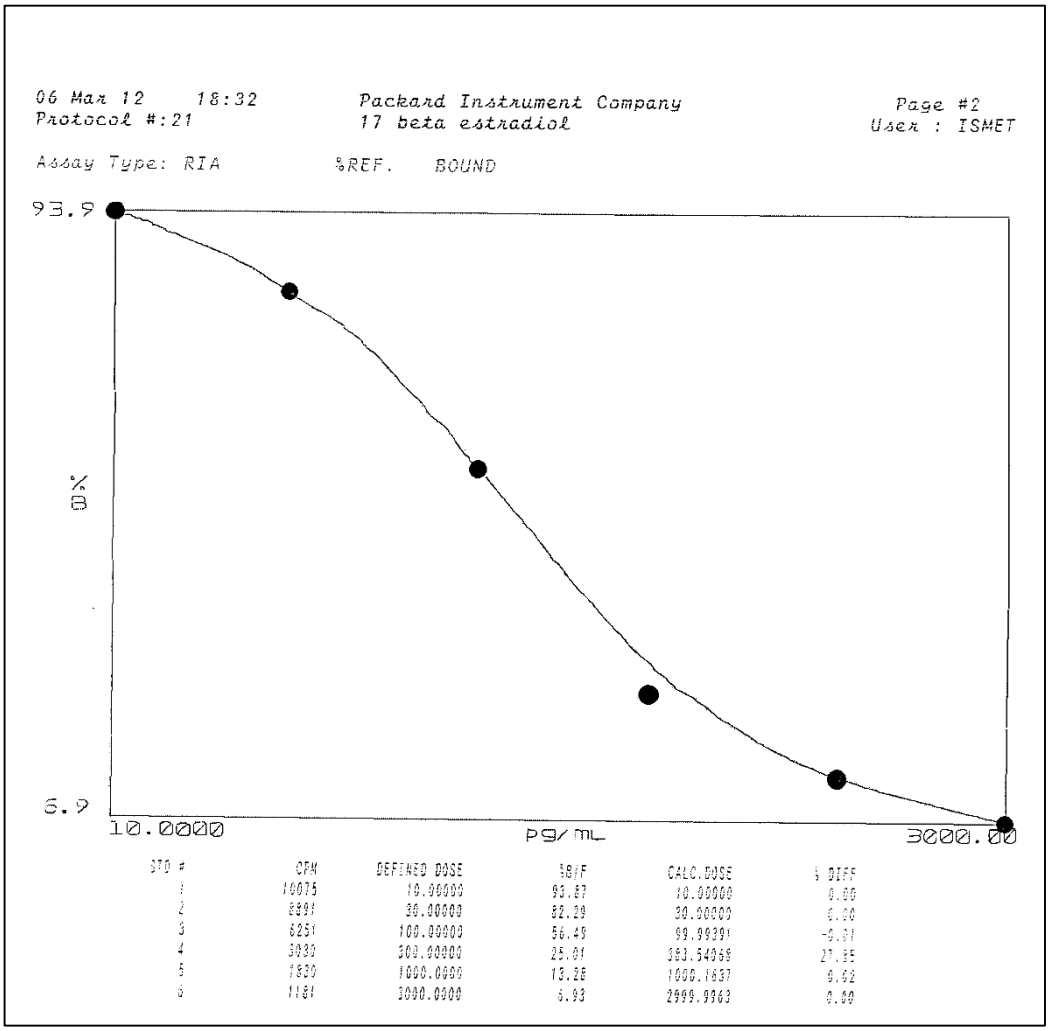

Figure 3.5: Calibration curve for the RIA measurements of E_2.

In order to eliminate the matrix effect, measured E_2 concentrations of patient samples (P and T) were normalized via substracting the measurement of the blank sample. Afterwards specific activity of aromatase ($U.mg^{-1}$) was calculated. Overall, we observed higher activity both in P and T than N, where the levels in T were slightly higher than N (Figure 3.6). The activity levels for each sample are shown in Figure 3.8. The highest specific aromatase activity was $3336{,}58.10^{-5}$ $U.mg^{-1}$ and observed in

the peripheral tissue. The lowest specific aromatase activity was $17{,}24.10^{-5}$ $U.mg^{-1}$ and observed in the tumor tissue (Figure 3.7).

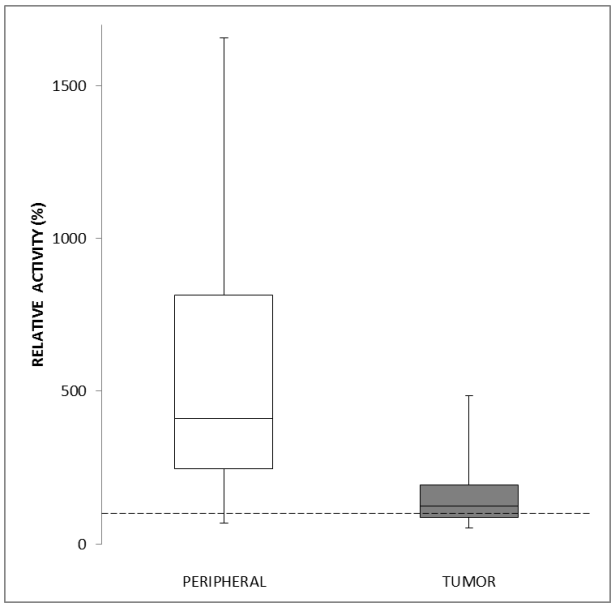

Figure 3.6: Relative aromatase activity levels for tumor and peripheral tissue samples among patients. The activity of healthy tissue samples which is represented here as the dotted line were used as reference. Calculations were made according to RIA measurements.

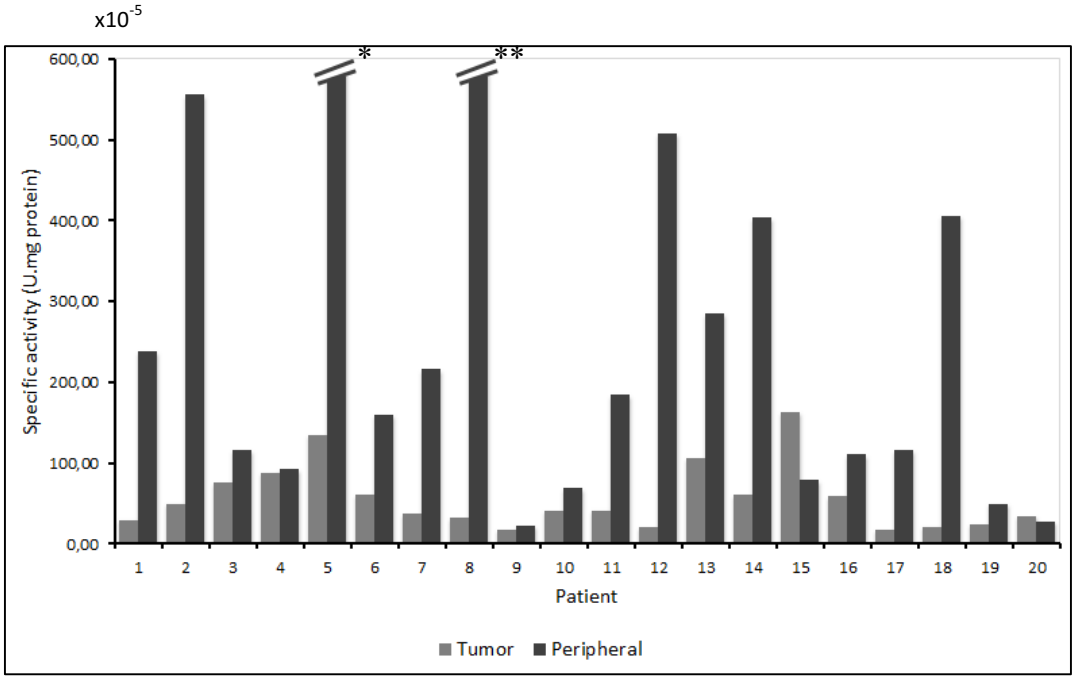

Figure 3.7: Specific activity of aromatase for tumor and peripheral tissue samples among patients. Calculations were made according to RIA measurements. (*) Activity of P for patient 5# was $3336{,}58.10^{-5}$ $U.mg^{-1}$; (**) activity of P for patient 8# was $1302{,}67.10^{-5}$ $U.mg^{-1}$.

More than half of the patients were observed to have higher activity in peripheral breast tissue compared to tumor tissue and the difference was statistically significant (Z= -3.51, p= 0.0004 [WSR]) (Figure 3.8-A). 70% of the measured aromatase activities were found to be consistent with CYP19A1 expression level results (Figure 3.8-B).

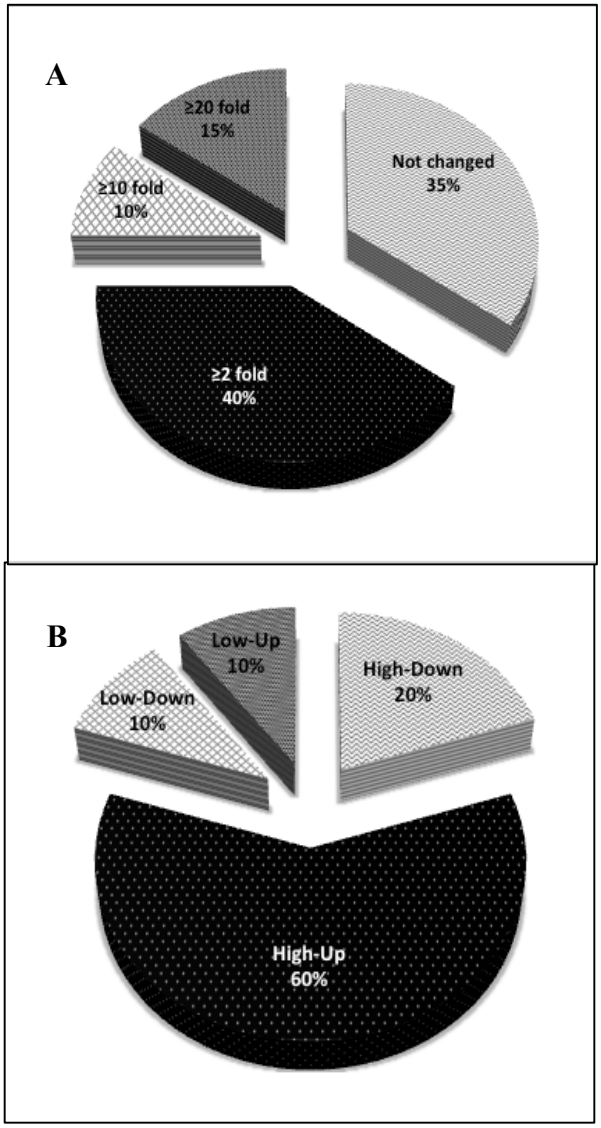

Figure 3.8: The distribution of aromatase activity levels calculated in regards of RIA measurements among the patients: A) P activity levels compared to T activity levels, the difference less than 2 fold was accepted as not changed; B) Crossmatched results of activity and expression levels for P compared to T. Low: Low aromatase activity, High: High aromatase activity, Down: CYP19A1 downregulated, Up: CYP19A1 upregulated.

When the expression levels of CYP17A1 and CYP19A1 were taken into consideration together, the fold difference of aromatase activity between P and T was higher in IP group (Figure 3.9). However the difference did not reach statistical significance. (p= 0.569 [MU])

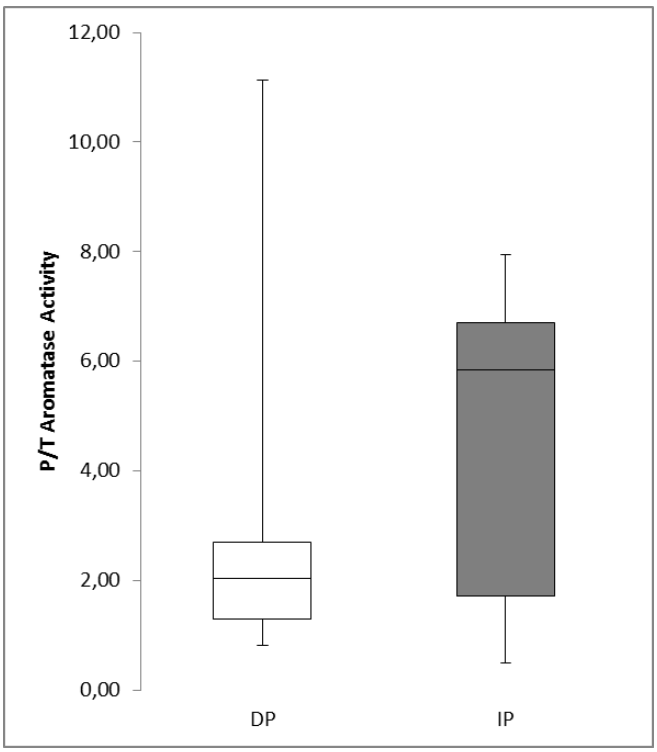

Figure 3.9: P/T aromatase activity levels calculated via RIA measurements among the estradiol decreasing (DP) and increasing (IP) expression pattern bearing groups. Upper outliers were not shown.

The significant associations of patient characteristics with specific aromatase activity, measured via RIA, in peripheral tissue compared to tumor tissue are summarized at Table 3.9. Oral contraceptive usage, presence of necrosis and absence of calcification were found associated with high activity levels in peripheral tissue, where other known factors were not.

Table 3.9: Tissue aromatase activitiy levels measured via RIA for different subgroups of patient characteristics. P < T: Peripheral tissue activity lower than tumor tissue activity, P > T: Peripheral tissue activity greater than tumor tissue activity.

Patient characteristics		P < T n (%)	P > T n (%)
Oral contraceptive use	Yes	0 (0.00)	2 (100.00)
	No	4 (22.20)	14 (78.80)
	p-Value (kappa)	0.00012 (0.054)	
Necrosis	Yes	1 (16.70)	5 (83.30)
	No	3 (21.40)	11 (78.60)
	p-Value (kappa)	0.006 (0.032)	
Calcification	Yes	2 (28.60)	5 (71.40)
	No	2 (15.40)	11 (84.60)
	p-Value (kappa)	0.022 (-0.102)	

3.5.2. LC-MS/MS measurements

In parallel to RIA measurement, we measured E_2 concentration of the same samples via LC-MS/MS.

Quantitative analysis was carried out using multiple reaction monitoring (MRM) mode and MRM transitions are shown in Table 3.10. Two MRM transitions per analyte were monitored (quantifier and qualifier) and a ratio of their peak areas was used for analyte confirmation. All of the samples were scanned for the quantifier, qualifier ions for each target analyte and MRM mass spectrums were obtained (Figure 3.10).

The calibration curves for the target compounds between 1-1000 ppb are shown in Figure 3.11. All calibration curves were generated using linear fit, no inclusion of the origin. All curves had linearity coefficients of at least 0.99 and showed good reproducibility and accuracy at the lowest levels. LOD and LOQ values for E_2 and TES were shown in Table 3.11.

Table 3.10: Mass Transitions of testosterone (TES) and estradiol (E_2).

	Compound	Percursor ion	MS1 Res	Product ion	MS2 Res	Dwell time (ms)	Fragmentor Voltage (V)	Collision Energy (V)	Polarity
Qualifier	TES	289.1	Wide	108.9	Wide	25	110	20	(+)
	E_2	255.1	Wide	133	Wide	100	90	14	(+)
Quantifier	TES	289.1	Wide	96.9	Wide	25	110	18	(+)
	E_2	255.1	Wide	158.9	Wide	100	90	12	(+)

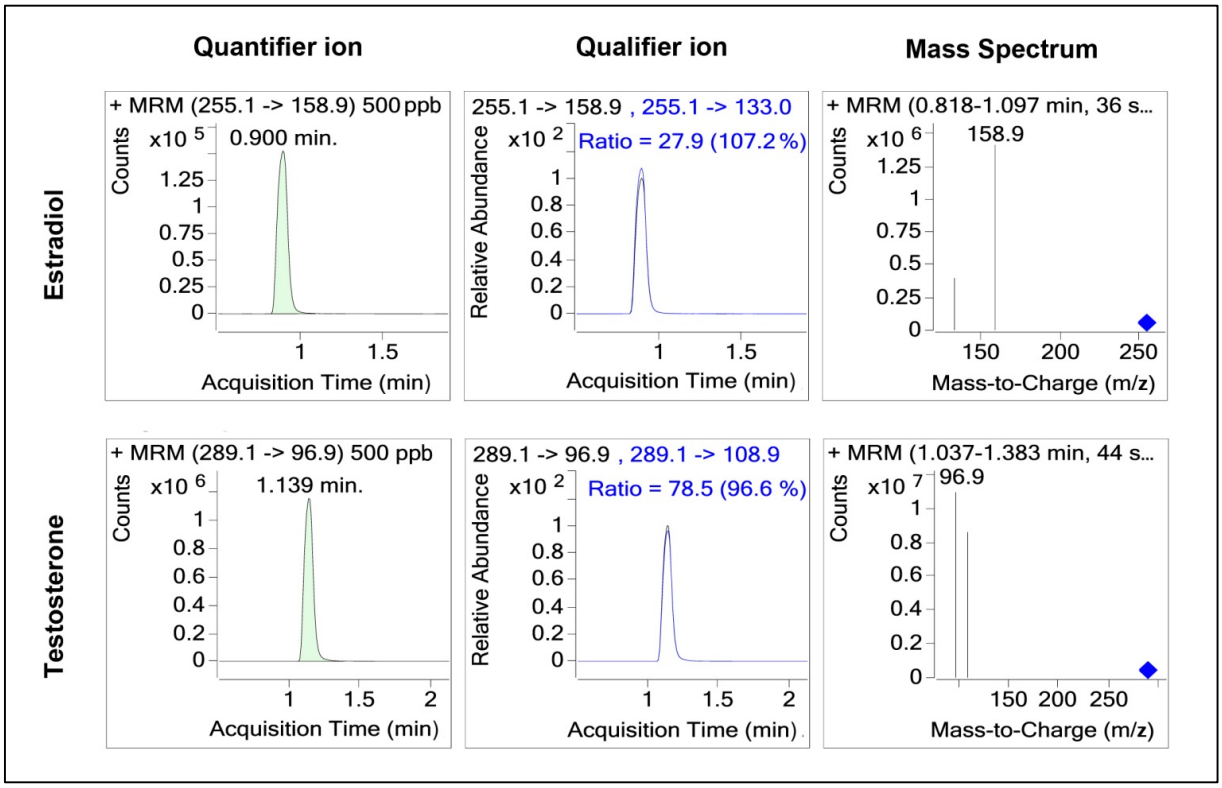

Figure 3.10: MRM mass spectrums of the quantifier and qualifier ions for each target analyte.

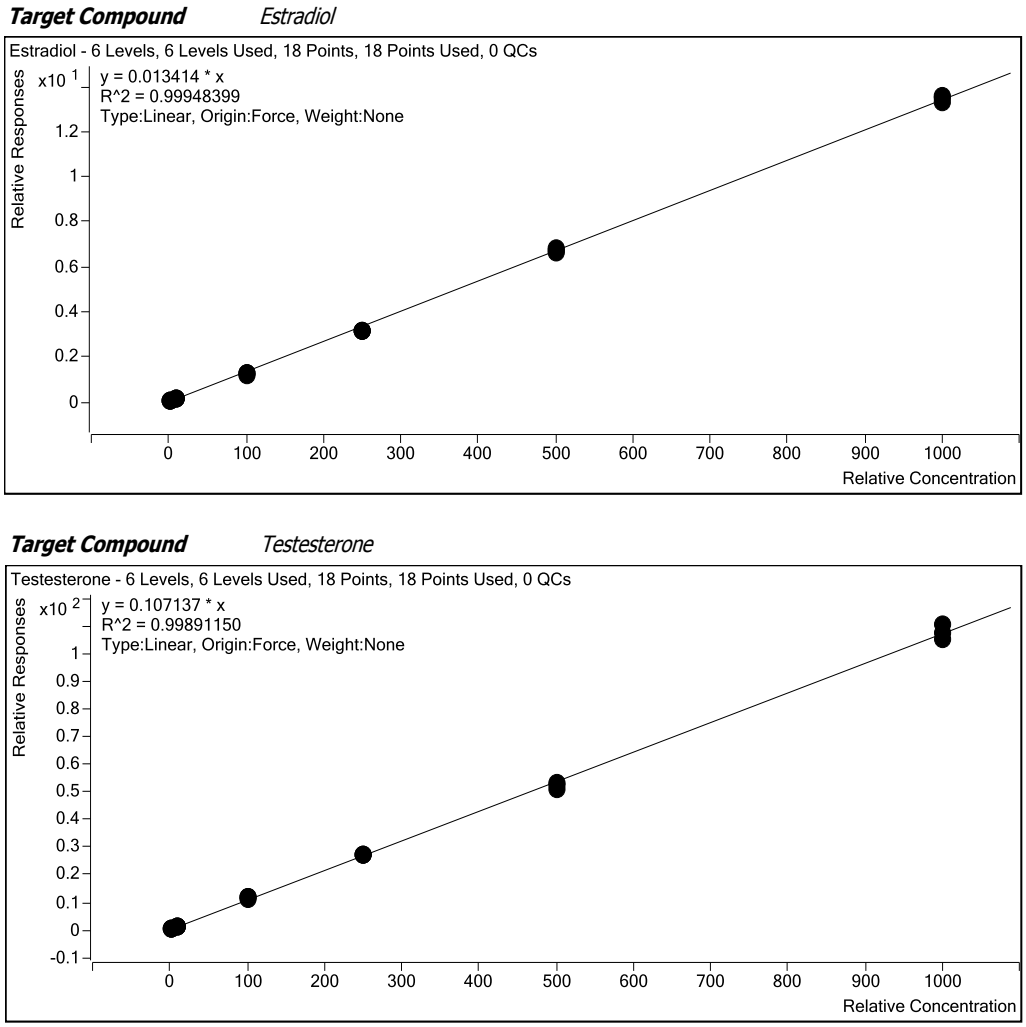

Figure 3.11: LC-MS/MS calibration curves of E_2 and TES.

Table 3.11: LOD,LOQ and accuracy values for E_2 and TES

	LOD (ppb)	LOQ (ppb)	Accuracy (Mean, ±SD)
Estradiol	9,29	28,16	100,91 ± 14,06
Testosterone	14,41	43,67	102,42 ± 6,21

In order to eliminate the matrix effect, measured E_2 concentrations of patient samples (P and T) were normalized via substracting the measurement of the blank sample. Chromatogram examples of blank, standart and unknown sample are shown in Figure 3.12.

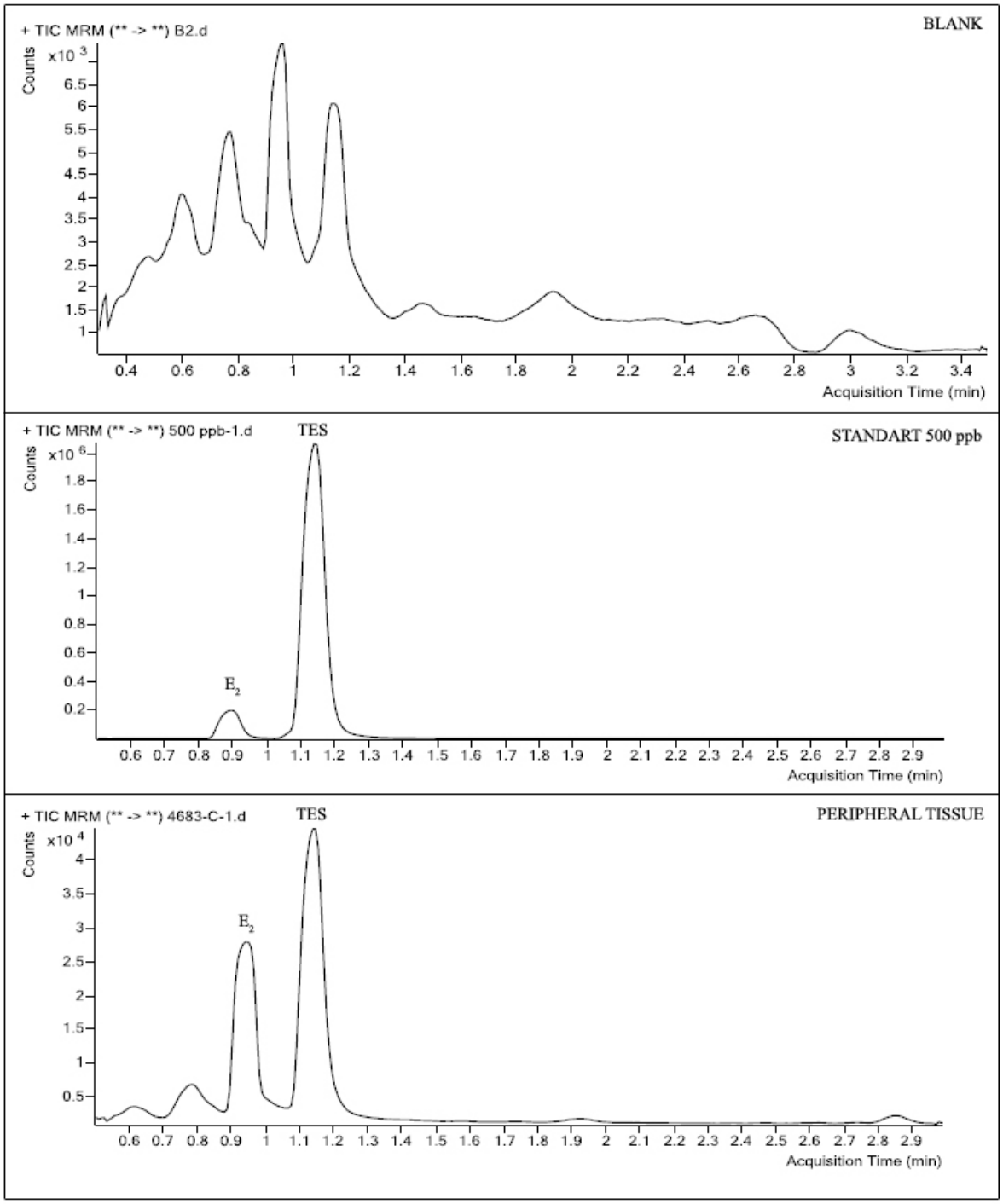

Figure 3.12: Chromatograms of the blank, standart and patient samples.

Overall, P had higher activity than N, on the other hand T showed approximately the same activity level with N (Figure 3.13). Specific activity of aromatase ($U.mg^{-1}$) was calculated for each sample, which are shown in Figure 3.14. The highest specific aromatase activity was 2582,40. $10^{-5}U.mg^{-1}$ and observed in the peripheral tissue. The lowest specific aromatase activity was $3,17.10^{-5}$ $U.mg^{-1}$ and observed in the tumor tissue.

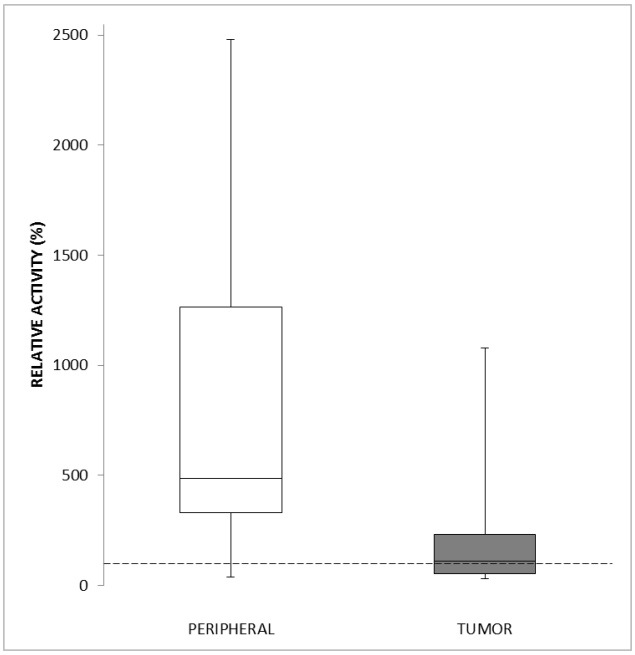

Figure 3.13: The relative aromatase activity levels for tumor and peripheral tissue samples among patients. The activity of healthy tissue samples which is represented here as the dotted line were used as reference. Calculations were made according to LC-MS/MS measurements. Upper outliers were not shown.

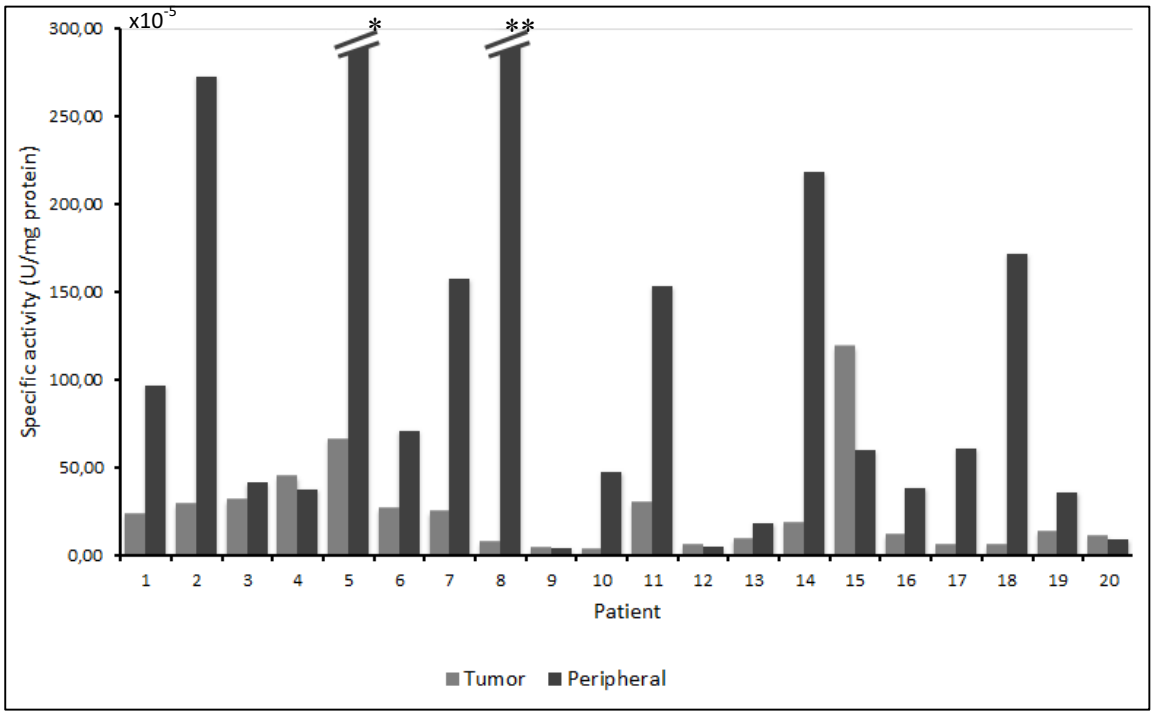

Figure 3.14: Specific activity of aromatase for tumor and peripheral tissue samples among patients. Calculations were made according to MS measurements. (*) Activity of P for patient 5# was 2582,40. 10^{-5} U.mg^{-1}. (**) Activity of P for patient 8# was 698,81. 10^{-5} U.mg^{-1}.

The calculated activity of aromatase was higher in peripheral breast tissue of 70% of the patients compared to their tumor tissue and the difference was statistically significant (Z= -3.17, p= 0.002 [WSR]) (Figure 3.15-A). 80% of the measured aromatase activities were found to be consistent with CYP19A1 expression level results (Figure 3.15-B).

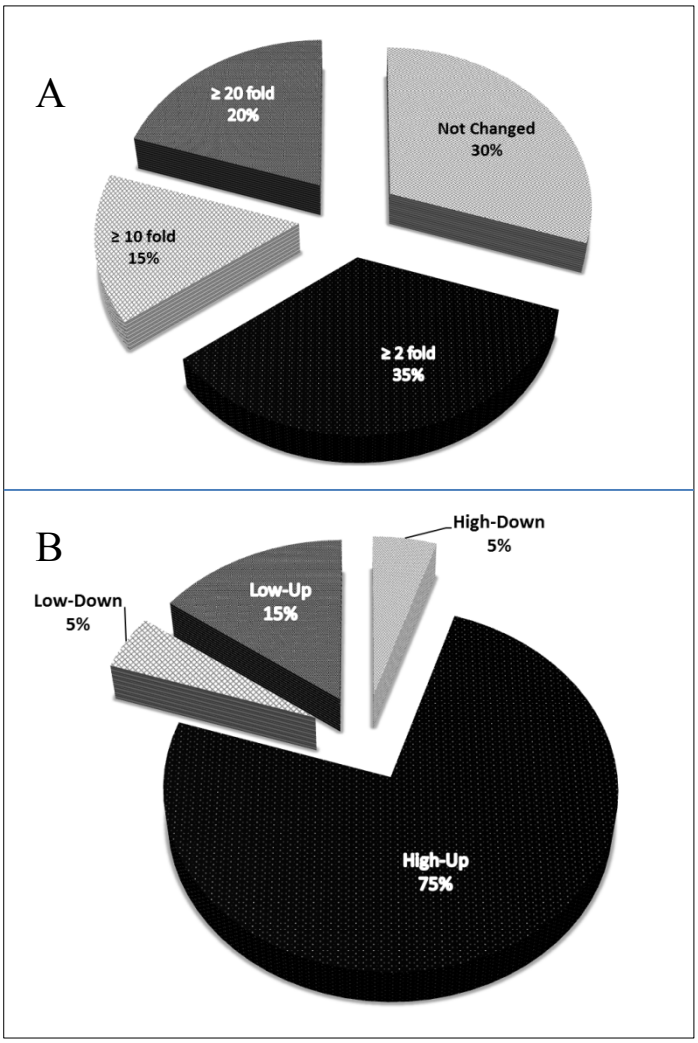

Figure 3.15: The distribution of aromatase activity levels calculated in regards of LC-MS/MS measurements among the patients: (A) P activity levels compared to T activity levels, the difference less than 2 fold was accepted as not changed; (B) Crossmatched results of activity and expression levels for P compared to T. Low: Low aromatase activity, High: High aromatase activity, Down: CYP19A1 downregulated, Up: CYP19A1 upregulated.

When the expression levels of CYP17A1 and CYP19A1 were taken into consideration together, the fold difference of aromatase activity between P and T was higher in IP group (Figure 3.16). However the difference did not reach statistical significance (p= 0.271 [MU]).

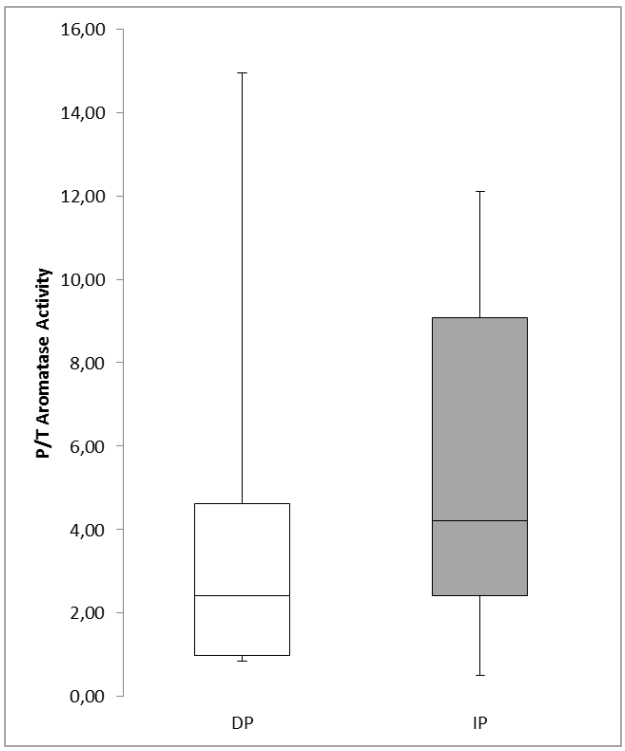

Figure 3.16: P/T aromatase activity levels calculated via LC-MS/MS measurements among the estradiol decreasing (DP) and increasing (IP) expression pattern bearing groups. Upper outliers were not shown.

The association of specific aromatase activity, measured via LC-MS/MS, and patient characteristics was also investigated. (Table 3.12). Oral contraceptive usage, presence of necrosis and absence of calcification were found significantly associated with high activity levels in peripheral tissue, where other known factors were not.

Table 3.12: Tissue aromatase activitiy levels measured via LC-MS/MS for different subgroups of patient characteristics. P < T: Peripheral tissue activity lower than tumor tissue activity, P > T: Peripheral tissue activity greater than tumor tissue activity.

Patient characteristics		P < T n (%)	P > T n (%)
Oral contraceptive use	Yes	0 (0.00)	2 (100.00)
	No	4 (22.20)	14 (78.80)
	p-Value (kappa)	0.0001 (0.054)	
Necrosis	Yes	1 (16.70)	5 (83.30)
	No	3 (21.40)	11 (78.60)
	p-Value (kappa)	0.006 (0.032)	
Calcification	Yes	1 (23.10)	6 (76.90)
	No	3 (15.40)	10 (84.60)
	p-Value (kappa)	0.012 (0.068)	

3.6 Lifetime Breast Cancer Risk Evaluation

We investigated the association of CYP17A1, CYP19A1 expressions and aromatase activity levels with individual lifetime (up to age 90) breast cancer risk of the patients. The International Breast Cancer Intervention Study (IBIS) Breast Cancer Risk Evaluation Tool (www.ems-trials.org/riskevaluator/) was used in order to estimate the risk (Figure 3.17). We chose this model according to the National Cancer Institute (NCI) reccomendations. Personal factors and family history of cancer information was used.

We detected a slight increase at the risk in patients who showed estradiol increasing pattern (Figure 3.18) and the difference was not statistically significant (p= 0.569 [MU]).

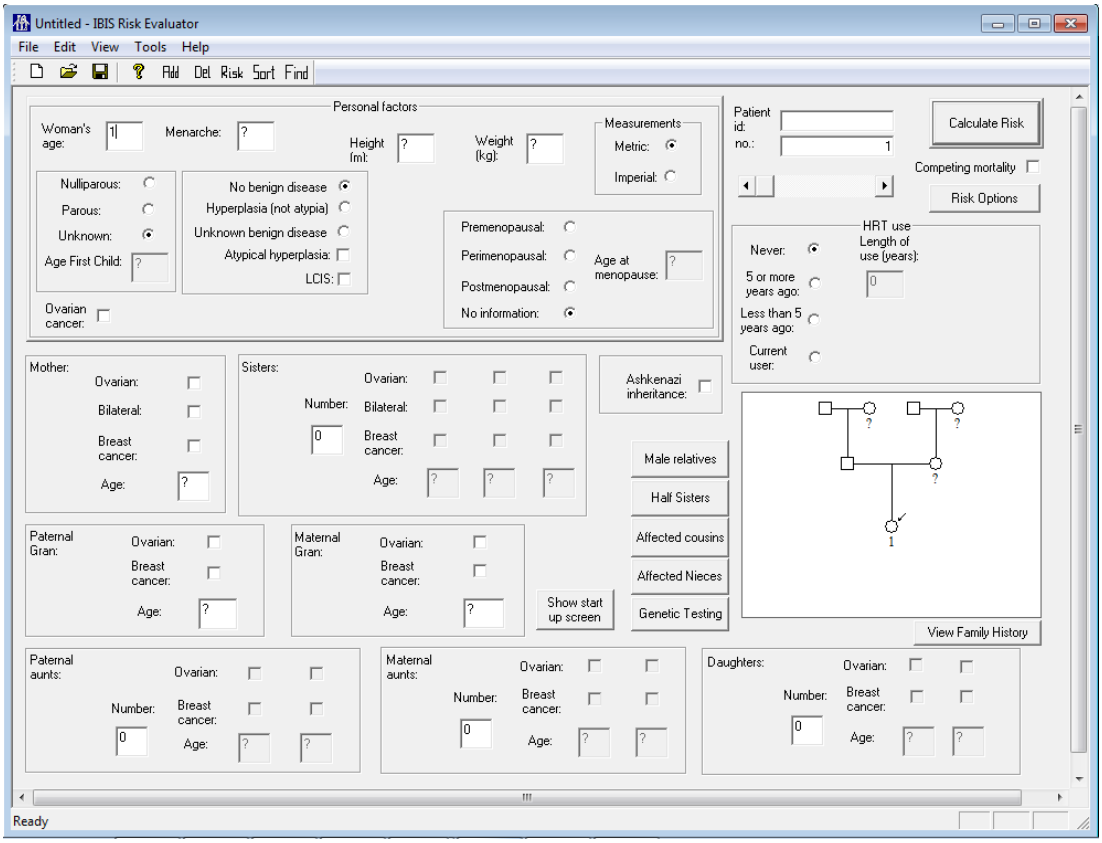

Figure 3.17: Main screen of the IBIS Breast Cancer Risk Evaluation Tool program.

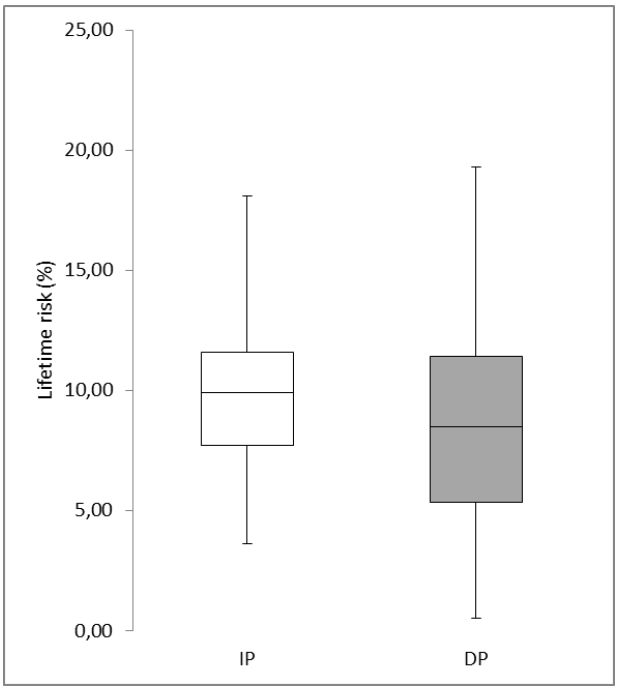

Figure 3.18: Lifetime breast cancer risk estimates among the estradiol decreasing (DP) and increasing (IP) expression pattern bearing groups .

We also observed the same risk escalation in cases which the specific activity of aromatase was higher at tumor tissue compared to its paired peripheral tissue (Figure 3.19). The difference was not statistically significant according to both measurement methods (p= 0.850; p= 0.409 [MU]).

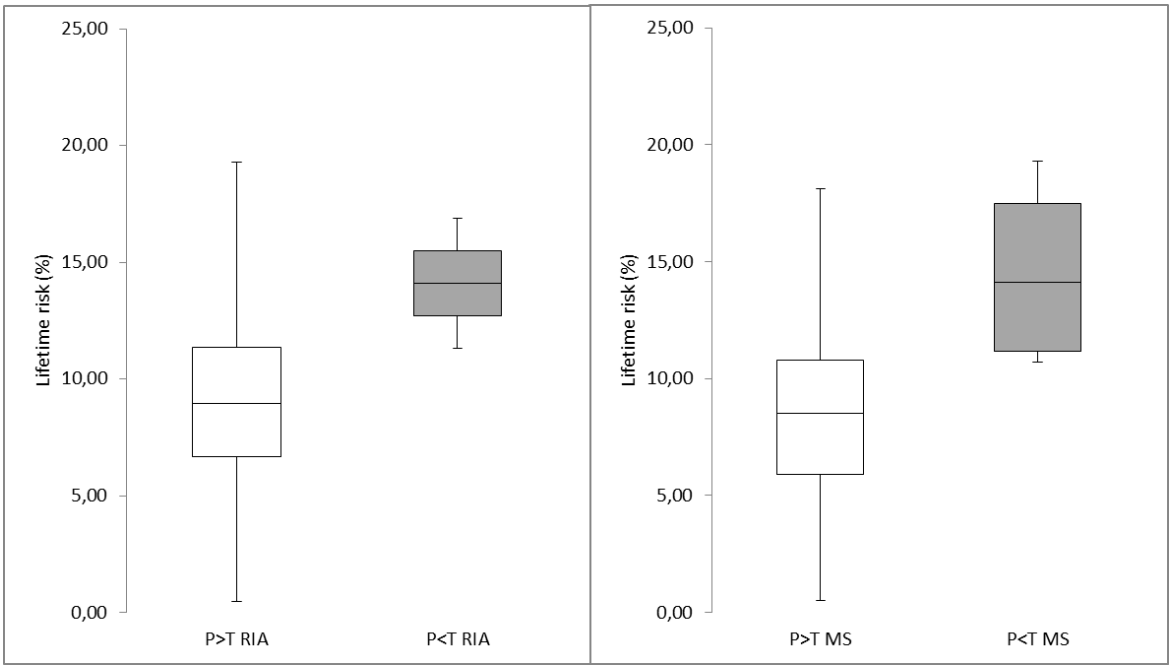

Figure 3.19: The effect of aromatase activity levels calculated via RIA and LC-MS/MS measurements over the lifetime breast cancer risk estimates

3.7 Comparison of RIA and LC-MS/MS Methods

We used two different methods for the measurement of the specific aromatase activity. Only two cases were measured differently, where higher activity levels in peripheral tissue were detected via RIA measurement (Table 3.13). An exact McNemar's test determined that there was no statistically significant difference in the proportion of aromatase activity measurements via RIA and LC-MS/MS (p = 0.500 κ= 0.737 [MN]).

Table 3.13: Crosstabulation table of LC-MS/MS and RIA measurements

		LC-MS/MS measurement	
		P<T	P>T
RIA measurement	P<T	4	0
	P>T	2	14

However the absolute values for specific aromatase activity calculated via RIA measurements were aproximately two times higher compared to LC-MS/MS measurement values (Figure 3.20).

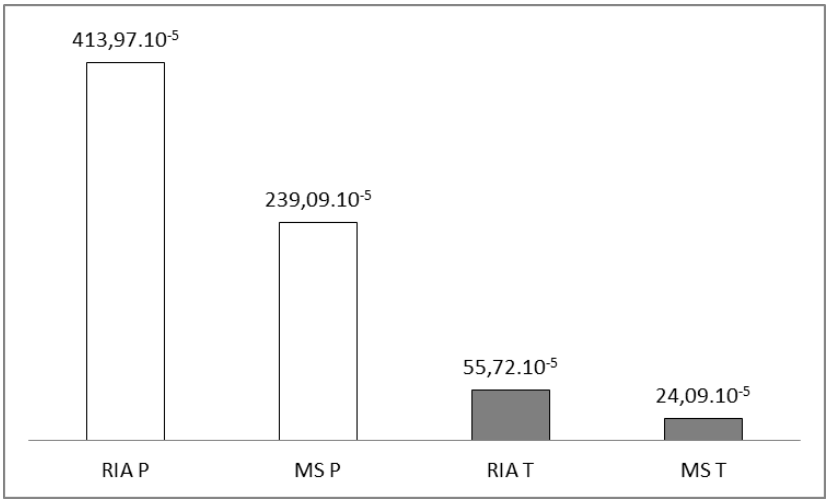

Figure 3.20: Average specific aromatase activity ($U.mg^{-1}$) levels of tissue types calculated via RIA and LC-MS/MS measurements.

4. DISCUSSION AND CONCLUSIONS

Local productions of estrogens in breast as well as the effects of estrogens on progression of breast tumors are attractive areas of research. Although a relationship between local estrogen production and the risk of breast cancer has been identified, at this point an accurate prediction of cancer risk in an individual is not possible. The variation in hormone levels within the different groups of people is yet to be understood. These results suggest additional mechanisms, such as up/down regulation of other enzymes involved in steroid synthesis pathway may influence intratumor levels of estrogens across individual tumor samples. Combined evaluations of these mechanisms including risk factors, such as family and reproductive histories, may lead not only to a more accurate assessment of risk in individual women but also to a better understanding of the role of estrogen in the pathogenesis of breast cancer (Clemons and Goss 2001, Li et al. 2005, Sidoni et al. 2003).

However, limited information is available on how these factors are related with gene expresion levels, which are considered as key factors in local estrogen production. Current study provides information about expression levels of CYP19A1 and CYP17A1 as well as aromatase activities within both tumor and and its peripheral adipose tissues compared to that in healthy breast tissue. The relationship between these expressions and activitiy status along with the breast health risk factors and clinicopathological parameters were also reported in order to investigate the effect of tumor progression.

Estrogen arises from two sources in a postmenopausal woman with breast cancer. First, estrogen that arises from extraovarian body sites such as subcutaneous adipose tissue and skin reaches breast cancer by way of circulation in an endocrine manner. Second, estrogen locally produced in breast cancer tissue makes an impact via paracrine or intracrine mechanisms.

Apparently, the major contribution for E_2 biosynthesis comes from the adipose tissue. Aromatase expression is almost exclusive to immature adipocytes and adipose

tissue related fibroblasts of the breast (Sasano et al. 2009, Geisler 2003). These cells convert progesterone to androgens, and furtherly to estrogens, in particular, estradiol via P450c17 (CYP17A1) and aromatase (CYP19A1) (Figure 4.1). Estrogens can diffuse throughout the tissue, especially the adipose tissue of the breast, and enter the breast duct, where they stimulate epithelial cell proliferation (Simpson and Brown 2013). Some however will enter the blood stream, circulate and then get taken up again by the adipose tissue, where they can mix with estrogen still present there and stimulate epithelial proliferation. Thus circulating estrogen levels will indeed be correlated with breast cancer risk, but this does not mean that they are the drivers of such risk; rather they reflect the local synthesis of estrogen within the adipose tissue, and within the breast in particular. Estrogens can act both directly or indirectly on human breast cancer cells to promote proliferation.

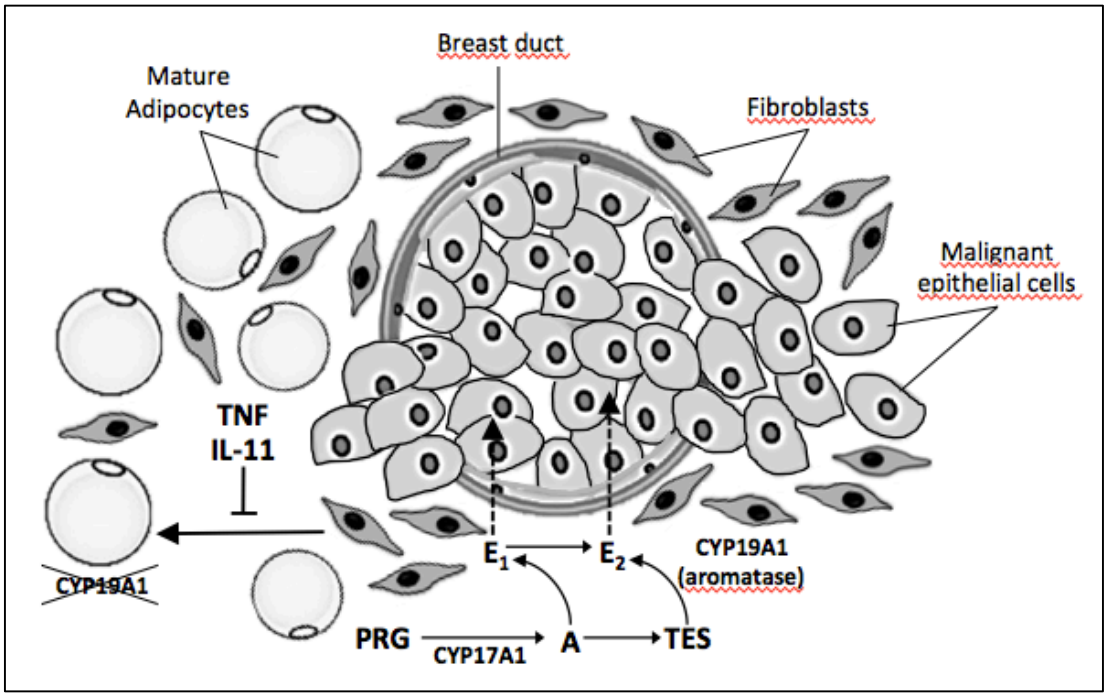

Figure 4.1: The roles of CYP17A1 and CYP19A1 in invasive ductal breast carcinoma progression.

Santen et al. (1997) have shown that stromal cells in breast carcinomas are the major source of estrogens in the tumor. There are other studies also found an elevation in aromatase expression in adipose stroma surrounding malignant breast epithelial cells which is regulated by complex cellular, molecular, and genomic mechanisms (Zhou et al, 2001; Agarwal et al, 1996). Independent studies were published from different laboratories where striking increases in aromatase enzyme activity and mRNA levels

signified in breast adipose tissue neighboring to tumor compared with those in distal adipose tissue or healthy breast adipose tissue (O'Neill et al., 1988; Bulun et al., 1993; Harada et al., 1993; Reed et al., 1993; Sasano et al., 1994; Utsumi et al., 1996; Zhou et al., 1996). Prominently, the overall aromatase expression in breast adipose tissue in mastectomy specimens bearing a breast tumor was significantly higher than that in benign breast tissue removed for reduction mammoplasty (Agarwal et al 1995, Agarwal et al 1996, Agarwal et al 1997). In the current study, CYP19A1 expression levels in peripheral tissues were nearly three times higher than the proximate tumor tissue (p=0.001 [WSR]), which is supporting the previous findings. In addition, higher aromatase activity in the peripheral tissues were observed compared to tumor tissues independent of measurement method. (p= 0.0004 [WSR] for RIA, p= 0.002 [WSR] for MS).

It is well-known that the estradiol concentration in plasma is significantly lower in postmenopausal compared to premenopausal women. Nevertheless, Pasqualini et al (1996), found that the levels of estradiol in breast cancer tissue were similar in premenopausal and postmenopausal women. Esteban et al (1992), found that 15 (40%) of 38 breast cancers demonstrated significant immunoreactivity for aromatase. Utilizing an assay for aromatase activity that quantifies production of tritiated water release from 1-tritiated-androstenedione, Lipton et al (1992) found measurable aromatase activity in 69% of 113 breast cancers. Another study performed by Yue et al (1998), who developed a model in which aromatase and sham-transfected MCF-7 cells were inoculated into ovariectomized nude mice, documented the pathologic significance of local aromatase activity in breast cancers in postmenopausal women. Heterotransplants in which aromatase-transfected MCF-7 cells were implanted in one flank and sham-transfected cells in the other flank demonstrated that the tumor weight was 7.6 fold larger and the estradiol concentration 3 to 4 fold higher in the aromatase transfected tumors. Our aromatase activity measurements detected the highest activity in peripheral tissue group and supports previous findings. Overall, peripheral aromatase activity was approximately 4 fold higher than the control (premenapausal) group. Furthermore, the tumor group showed almost the same level of aromatase activity with controls (Figure 3.6 and 3.12). Thus, we can speculate that the source of local estradiol, which plays a major role in proliferation of malignant epithelial breast cancer cells, depends on the power of local aromatization activity.

Local steroid biosynthesis and metabolism of exogenous ligands adds further complexity to the balance between estrogen and androgen hormone action, and it is now recognized as a crucial aspect contributing to the progression of estrogen driven breast carcinomas (Risbridger et al, 2010). For example, estrogen receptor-α (ERα) is considered as the primary proliferative and survival stimulus in breast epithelial cells. However, this stimulating effect compansated by androgen action through the androgen receptor (AR), which has direct inhibitory effects on cell survival and can inhibit the proliferative actions of estrogen. The expression of progesterone receptor (PR) increases with this estrogen action. Progresterone action can both stimulate and inhibit the proliferation in breast cancer cells (Figure 4.2). Some evidence suggests that androgen action might also inhibit the PR signalling pathway but this remains uncertain (Risbridger et al, 2010). The impact of CYP17A1 is becoming important in this context where its activity determines the fate of progesterone. Therefore, trying to explain estardiol's effect on estrogen driven breast carcinomas based on only local aromatase expression and activity may not reflect the reality. Previously our group showed how the different allele combination of CYP17A1 and CYP19A1 genes might effect on breast cancer risk (Tuzuner et al 2010). In current study, we assumed combination of upregulated and unaltered CYP17A1 and CYP19A1 expression levels to increase estrogen production. Although, this pattern coralates with higher aromatase activity levels in peripheral tissues compared to tumor tissues, it did not reach statistical significance (Figure 3.9 and 3.15). The expression levels of other critical enzymes such as 17β-HSD and 5α-reductase should also taken into consideration in order to get results that are more reliable.

Postmenopausal women's ovaries cease to synthesize estrogens; however, the risk of breast cancer continues to increase with age. The main reason is the continuous exposure of estrogen to breast tissue in postmenopausal women (Zhu and Coney 1998). Although, we did not observe an effect of age over expression levels or aromatase acivity in our relatively small (n= 20) study population.

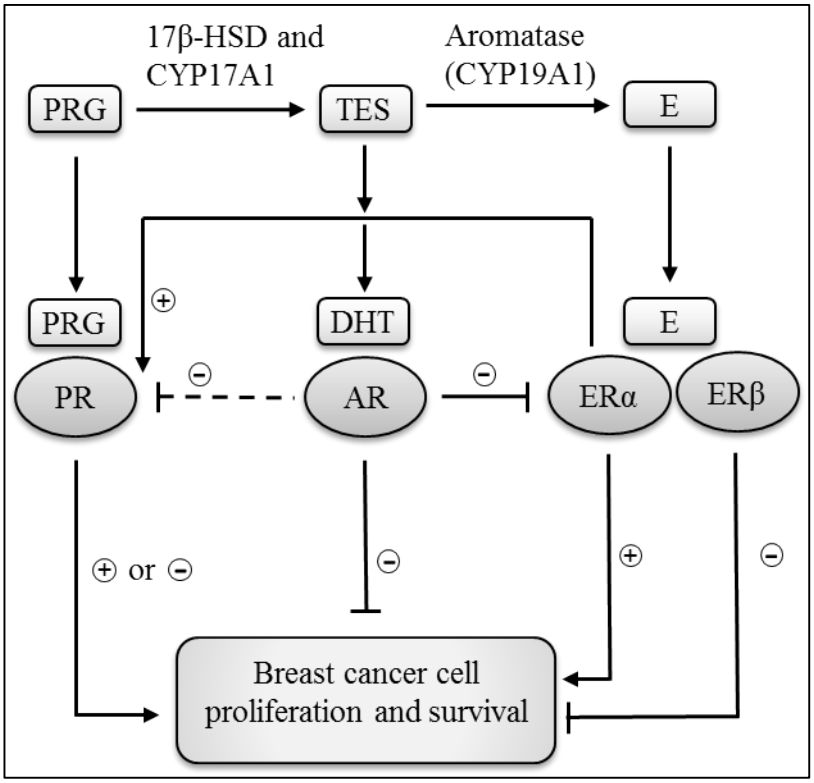

Figure 4.2: Hormone interactions in breast epithelial cells. The dashed line represent a putative inhibitory effect. PRG: Progesterone; TES: Testosterone, E: Estrogens; DHT: 5α-dihydrotestosterone.

Miyoshi et al. (2003) observed higher CYP17A1 mRNA levels in small tumors (≤ 2 cm), suggesting that CYP17A1 mRNA upregulation might play some role in the early stage of tumor development. In the current study most of the patients (75%) had late onset breast cancer, which may be associated with unaltered CYP17A1 expression in peripheral tissues and slight down regulation in tumors. On the contrary, in peripheral tissues at the early onstage of the disease, when both CYPs were together, they appeared to decrease of local estrogen production via regulating the levels of expression (p=0.021 [MN]). It was also possible to observe this via histological grade parameter where high-grade tumor tissues were found down regulated (p= 0.012 [MN]), while grade I tumor tissues exhibited higher levels of CYP17A1 expression (p= 0.013 [MN]). Thus, it can be speculated that tumor tissues are more active for estrogen production than peripheral tissues in the early phases of breast cancer and later on production site shifts to peripheral fibroblasts.

The most significant prognostic factor for women with breast cancer is axillary lymph node invasion status (Donegan 1997). Nodal metastases double the risk of distant disease and the presence or absence of axillary lymph node metastases is also

a determining factor for the use of systemic adjuvant chemotherapy (Hortobagyi and Buzdar 1995). Here CYP19A1 in neighboring adipose tissues were highly expressed in all cases with positive axillary invasion (p= 0.012 [MN]), thus it may be suggested as an additional parameter for such decisions like adjuvant chemotherapy.

Upon outgrowth of their oxygen supply, emerging tumors develop hypoxic and eventually necrotic regions believed to be the direct result of chronic ischemia caused by vascular collapse when the rate of tumor cell growth exceeds that of angiogenesis. Necrosis in invasive carcinoma of the breast has been correlated with the concomitant angiogenesis, development of high vascular density, and increased levels of focal macrophage infiltration driven by chemotactic factors. Necrosis at the tumor center is a common feature of aggressive breast cancer and has been associated with poor prognosis. Our data showed association of higher aromatase activity in peripheral tissue compared to its paired tumor tissue among the cases with necrosis (Table 3.9 and 3.12). Invasive ductal breast carcinoma cases so closely mimicking ductal carcinoma insitu (DCIS) with central necrosis that on initial morphological analysis these foci of tumors were labeled as DCIS (high grade, comedo). However on further histological work up and by using IHC for myoepithelial markers it was later confirmed that these were foci of invasive ductal carcinoma breast with central necrosis (Pervez and Khan 2007). Evaluating of aromatase activity levels with necrosis status may be an asset for discrimination of such cases.

The presence of calcification clusters is an important sign for the detection of early breast carcinoma. An early sign of 30–50% of breast cancer detected mammographically is the appearance of clusters of fine, granular microcalcification, and 60–80% of breast carcinomas reveal calcifications upon histological examinations (Cheng et al 2003).We observed higher aromatase activity in peripheral tissue compared to tumor tissue in the 77% of the cases with calcification (p=0,012). The underlying molecular mechanism was not clear, thus the effect should be investigated furtherly with greater study groups.

The expression of ER, PR and HER2 are key in determining prognosis and management. Especially both ER positive and PR positive (rather than only ER positive) tumors may respond better to hormonal therapy. In addition, absence of PR expression in primary breast cancer is strongly and independently associated with

poor prognosis (Purdie et al. 2014). Association between weak PR and low expression levels of both CYP17A1 and CYP19A1 in tumor tissues that we observed in this study, showed that these expression levels could be taken into consideration to identify patients who might benefit additional adjuvant chemotherapy, extended endocrine therapy and/or treatments targeting growth factor receptor pathways. Although there are preferred regimens in hormone treatment, such as aromatase inhibitors or ER blockers (Fisher et al. 2005), predicting which treatment could be most profitable for a particular patient is yet unclear. Evaluating the expressional levels of certain CYPs, due to their key role in estrogen synthesis, may be valuable for selecting the right treatment in addition to the conventional approach.

CYP19A1 up regulation in peripheral tissues is associated with familial incidences of cancer, breast cancer in particular ($p= 0.039$, $p= 0.004$ [MN]). Accumulation of different genotypes for different mutations of CYP19A1 may affect expression levels, thus altering aromatase activity and and subsequently affecting the endogenous estrogen levels (Surekha et al. 2014, Tüzüner et al. 2010). However, we did not observe a significant effect of this factor to the aromatase activity difference among the tissue groups.

Expresion patterns of CYP17A1 and CYP19A1 may influence the degree of estrogen exposure on breast epithelial cells. Since the effects of the various hormonal risk factors might dependen on the expression levels in different tissues, this possible diversity was of interest. Patients bearing “high risk” factors such as early onset of menstruation, nullparity and being older than 50 years old produced low levels of both expression and activity for CYP19A1 in P and T, which was not in agreement with the previous findings (Clemons and Goss 2001). However late age at pregnancy and the association with upregulation of aromatase expression in P, supported previous results ($p=0.039$ [MN]) (Russo et al. 2005, Britt et al. 2007). We also observed higher aromatase activity in P compared to T in this group, although the kappa value is negative. Pregnancy may have direct effect on immature adipocytes, causing them to differentiate and maturate which can no longer express aromatase. Nevertheless, tumor cells prevent these fibroblasts to become mature adipocytes, therefore women who had children after 30 years of age are probably more prone to this effect.

The growing public awareness of breast cancer and its risk factors, coupled with the availability of medical and surgical risk reduction options, has led to many women consulting their doctors regarding their breast cancer risk. Multiple prediction models have been developed to assist with breast cancer risk prediction efforts. We aimed to investigate if there may be an association between the outcome of a such model and expression and activity levels. The Tyrer-Cuzick model (IBIS) (Tyrer et al 2004), was used in our study since it is the only risk assessment model that explicitly accounts for differences in breast cancer risk, given the presence of atypical hyperplasia as well as extensive family history information, endogenous estrogen exposure, and benign breast disease (Amir et al 2003). We observed that the higher the lifetime risk were associated with estradiol increasing expression pattern. However, the lifetime risk was found to be decreased in patients who had higher specific activity of aromatase at peripheral tissue compared to its paired tumor tissue (Figure 3.18). The possible explanation of this discrepancy is the unequal distribution of the patients between groups where only few patients have higher specific aromatase activity in their tumor tissue compared to peripheral tissue (RIA n=2, LC-MS/MS n=4) (Table 3.12). CYP17A1 and CYP19A1 expression alterations may be considered as factors for such risk model in the future if their effect significantly exhibited via studies with greater sample sized.

In current study we have developed two different method, RIA and LC-MS/MS based, for the measurement of the specific aromatase activity of the microsomal fraction extracted from the breast adipose and tumor tissue. One of the aims in this study was to determine whether similar RIA and LC-MS/MS results would be attained or not. Although the RIA overestimated E2 concentrations (approximately 2 fold) (Figure 3.19) matched to LC–MS/MS; the calculated aromatase activity ratio of peripheral adipose and tumor tissue was aproximately same in both methods. The observed discrepancy between methods for the samples was most likely due to the sample matrix and the specificity of the RIA kit antibody. Many studies have indicated that RIA exhibits poor specificity for steroids with several interferences and severe matrix effects. Antibodies used for RIA can cross-react during the recognition of steroids, which are present in low concentrations in complex biological fluids (Hoofnagle and Wener 2009, Stanczyk and Clarke 2010, Stanczyk et al 2007). On the other hand, the primary matrix effect associated with LC-MS/MS

methods is ion suppression or enhancement caused by the co-eluting matrix components. This effect can be caused by both inorganic and organic endogenous substances, including salt, carbohydrates, amines, urea, lipids, peptides, and metabolites (Antignac et al 2005). A possible explanation is that the matrix compounds compete with analyte for the limited charge on the droplet surfaces and, thus, affect ionization of analyte. Another possible explanation is that interfering compounds increase the droplet's viscosity and surface tension, thereby decreasing solvent evaporation rate. As a result, a lesser amount of analyte is able to reach the gas phase. It has also been suggested that nonvolatile materials in the matrix can decrease the rate of droplet formation though coprecipitation of the analyte and, therefore, prevent droplets from reaching the critical radius required for gas phase ions to be emitted (Chiu et al 2010).

Another drawback of RIA observed in current study was the difficulties in optimization of a commercial kit for tissue extract samples. In addition the short shelf life and the special requirements for the storage and waste disposal could be reffered as disadvantages, hence the method depends on a radioactive compound (^{125}I).

It appears, the in-house LC-MS/MS method more advantegous compared to RIA. Although we observed the possible ion suppression related loss of sensitivity compared to RIA, after optimization of the conditions and the proper calibration of the analytes, we managed to detect and quantify the formed E_2 via microsomal fraction of the tissue extract with higher selectivity and improved signal-to-noise ratios. The developed method did not require derivatization of the analytes (E_1, E_2 and TES), therefore faster and simpler and displays high selectivity and accuracy compared to RIA. Considering the tandem mass spectrometry hyphenated to liquid chromatography separation systems has developed to an important technology in clinical chemistry (Vogeser and Seger, 2008), the current method has the potential to be further developed to a commonly applied high-throughput technique for aromatase activity measurement.

Lifetime exposure to estrogen and other physiological factors, including environmental exposures, could play a critical role in the etiology of breast cancer. Finding effective therapy approaches for cancer is crucial, as is early diagnosis. The ultimate goal will be to have accurate molecular profiling of patients with estrogen

driven breast cancer that can personalise treatment pathways, which in turn will enhance the effectiveness of current strategies. In conclusion, this study suggests that evaluation of various clinicopathological and disease risk factors along with the expression levels of CYP17A1 and CYP19A1 and the aromatase activity levels at breast tumor microenvironment might help clinicians to decide on treatment startegies and diagnosis for individual cases, particularly with postmenapausal status. However, future studies must be conducted using greater sample size and addition of other key enzyme activities evaluations in streidogenesis pathway which effect local estrogen levels for confirmation and gettting more strong and reliable results.

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